The Function and Regulation of Sleep in *Drosophila melanogaster*

Vanessa Maria Hill

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
under the Executive Committee
of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2018

Abstract

The Function and Regulation of Sleep in *Drosophila melanogaster*

Vanessa Maria Hill

A key feature of sleep is reduced responsiveness to the environment, which puts animals in a particularly vulnerable state; yet, sleep has been conserved throughout evolution, indicating that it fulfills a vital purpose. A core function of sleep across species has not been identified, but substantial advances in sleep research have been made in recent years using the genetically tractable model organism, *Drosophila melanogaster.* While a standard approach in sleep research is to study the effects of short-term sleep deprivation on an animal, tools are now available to genetically manipulate sleep amount in the fruit fly. In particular, a number of short-sleeping *Drosophila* mutants have been identified that model the long-term sleep restriction that is widespread in modern society. This thesis describes a body of work in which short-sleeping *Drosophila* mutants, as well as other genetic and pharmacological tools, were used to shed light on the function and regulation of sleep.

Table of Contents

List of Figures

Acknowledgements

I would like to thank Mimi Shirasu-Hiza for her invaluable training and mentorship. Mimi has offered me endless advice, fresh perspectives, and the independence to pursue my own ideas, even if they end in failure (see Chapter IV). Her unrelenting optimism and words of encouragement have raised my spirits and pushed me to persevere many times throughout graduate school.

I would also like to thank my thesis committee members, Wes Grueber, Julie Canman, and Adam Ratner, for their support, advice, and many insights over the years, which have had a constructive impact on my research. Special thanks to Julie Canman for her valuable contributions (including the cutest snoozing fly) to the work in Chapter II. Thank you also to my outside defense committee member, Julie Williams, for kindly serving on my committee.

Thank you to all the members, past and present, of the Shirasu-Hiza lab, who helped to create a constructive and harmonious lab environment. In particular, thank you to our army of undergrads—Gunter Sissoko, Ifeoma Irobunda, and Stephen Leong—for their work on this project, and also thank you to our postdocs, Matt Ulgherait, Emily Marcinkevicius, and Becky Delvanthal, for many insightful discussions.

Lastly, thank you to my parents, Ken and Jeanne, my brother, Michael, and my husband, Jeremy, for their unwavering love and support, and their efforts to keep me sane.

Dedication

To my parents. Thank you for paving the way.

Chapter I: Introduction

Though humans have been speculating on sleep since ancient times, it still remains one of our most mysterious behaviors. Ancient people were intrigued by sleep because its main behavioral features—lack of movement and failure to respond to the environment—parallel death. In Greek mythology, sleep is personified by Hypnos who lives in the underworld with his twin brother, Thanatos, the god of death [1]. The idea that sleep represents a death-like state persisted even into the 1800's, with Robert MacNish's "Philosophy of Sleep," in which he explains that "sleep is a temporary metaphysical death" [2].

Scientific explanations for the cause of sleep arose during antiquity, but were largely unquestioned until modern day. The first sleep mechanism was proposed in 6th century BCE by Alcmaeon who believed that sleep occurs when blood retreats to the internal organs [1]. Two centuries later, Aristotle hypothesized that stomach vapors rise to the brain during digestion, and the cooling of these vapors triggers sleep [1]. Remarkably, variations of the stomach vapor theory survived for over two millennia before the birth of neuroscience and the discovery of new technology led to a surge of sleep research in the 20th century.

The idea of a hypnotoxin, or a sleep-inducing toxin that accumulates in the brain during wake and interferes with neuronal function, emerged in the early 1900's with the work of Ishimori [3] and Legendre and Piéron [4]. Both groups independently discovered that cerebrospinal fluid extracted from sleep deprived dogs promptly induces a deep sleep when injected into other dogs. These findings sparked the quest for a sleep-

promoting factor that researchers believed would unearth the mechanisms underlying the function and regulation of sleep.

Over the past century, considerable advances have been made in our understanding of sleep. The development of the electroencephalogram (EEG) led to the discovery of different sleep states and stages; the discovery of the circadian system shed light onto one branch of sleep regulation; and researchers began to identify genes that influence sleep. Despite this rapid progress in sleep research, the function of sleep and the second branch of sleep regulation (sleep homeostatic mechanisms) still remain unclear.

Since our transition into industrialized society, artificial lighting and 24-hour access to entertainment, food, and stimulants has made insomnia and sleep restriction a growing problem [5]. Health consequences such as diabetes and heart disease, two of the top ten leading causes of death in the US [6], have been linked to sleep restriction [7,8]. Lastly, sleep disturbances have been shown to precede the development of neurodegenerative diseases such as Alzheimer's Disease [9], another leading cause of death. Thus, there is a great need to further our understanding of sleep, and for the first time in history, there is a wealth of tools available to do just such.

This introductory chapter will address the universality of sleep, with emphasis on sleep in the fruit fly. It will summarize our current understanding of sleep regulation, present prevalent theories on the function of sleep, and highlight a somewhat forgotten theory: The Free Radical Flux Theory of Sleep. Chapter II will present data in support of a bi-directional relationship between sleep and ROS, and Chapter III will dive deeper into the total body of work on this topic, providing additional data in support of Chapter II

while also addressing experimental challenges and confusing data. A side project on the effect of social isolation on immunity will be presented in Chapter IV. Chapter V will include a more detailed discussion and future directions.

The Universality of Sleep

Defining Sleep

While immobility is the most obvious behavioral characteristic of sleep, lack of movement does not distinguish a sleeping animal from one that is simply resting or in another physiological state, such as hibernation. In mammals and birds, sleep has been defined by characteristic changes in brain activity that can be recorded by an EEG. For instance, the deepest stage of sleep, called slow wave sleep, occurs during non-rapid eye movement (NREM) sleep, and features prominent delta waves in the 0.5-4.5 Hz range [10]. However, EEG recordings cannot easily be done in some species, including invertebrates. Thus, starting with Piéron's observations in 1913, and with several additions from others over the past century, scientists have developed a set of behavioral criteria that define sleep. An animal is said to sleep only if it 1) demonstrates reversible immobility, 2) assumes a sleep-specific posture, 3) exhibits increased arousal threshold, or reduced reaction to a stimulus and 4) shows a sleep rebound, or a recovery period of longer and/or deeper sleep after sleep deprivation [11,12]. The behavioral criteria of sleep have been helpful in determining whether sleep exists in non-mammalian species.

Sleep Across Species

One feature of sleep that makes it particularly intriguing is its seemingly ubiquitous nature. This is especially true when considering how dangerous sleep behavior can be. A sleeping animal is vulnerable to predators and other dangers in its environment for many hours each day. The fact that animals have evolved such a behavior suggests that sleep must fulfill a function that is fundamental to life. This notion supports the idea that is shared by many, but not all sleep researchers, that there is a core function of sleep across all animal species. If sleep truly is crucial to animal life, then we would expect to see sleep behavior in all animal species. Whether or not this is the case is still under some debate.

Prior to 2000, sleep research focused largely on mammals and birds, despite the fact that sleep behavior had been described in many simple vertebrate and invertebrate species by that time. Campbell and Tobler had examined over 200 studies on various species and determined, based on behavioral criteria, that sleep is present in fish, reptiles, amphibians, and invertebrates such as the cockroach [11]. Evidence of a sleep state in bees [13] and scorpions [14] had also been reported. In the last two decades, better technology has provided more extensive evidence of sleep in organisms that were once thought to be sleepless: slow wave sleep has been reported in crayfish [15], a change in brain state during sleep was demonstrated in fruit flies [16], and varying sleep intensity, indicative of sleep stages, has been shown in both honey bees and fruit flies [17,18]. As such, sleep research has now expanded into model organisms such as the zebrafish [19], fruit fly [20,21], and even the roundworm [22].

Nonetheless, some researchers have argued against the presence of sleep in all species [23]. Often, the bullfrog is cited as an animal that does not sleep; yet, the only evidence on this comes from a single study conducted in 1967, in which electric shock was used as an arousal stimulus, and an increased arousal threshold could not be shown during quiescence [24]. Other animals that have been referenced as proof that sleep is not universal include a species of coral reef fish that engages in “sleep swimming,” which involves continuous movement of the fins while the fish stays in one place [25]. Sleep swimming may be necessary to prevent hypoxia of the coral colonies within which these fish sleep [25]. While the fish lack complete immobility during sleep swimming, they are much more likely to be caught by predators during this behavior, suggesting that they do indeed exhibit an increased arousal threshold indicative of a sleep state [26]. Sleep swimming is also present in the dolphin, another species in which the presence of sleep has been questioned.

Dolphins exhibit circular swimming, a behavior during which they have been shown to be less responsive to stimuli [27]. Circular swimming coincides with “unihemispheric sleep,” during which only half of the brain produces the slow waves characteristic of deep sleep at one time [28]. This strategy is thought to allow the animals to surface for air during sleep, and is shared by other aquatic mammals such as porpoises and whales [29]. Unihemispheric sleep is also common across numerous avian species, in which it likely serves to reduce the risk of predation during sleep [29]. Behavioral observations suggest that certain reptilian species may also rely on unihemispheric sleep to watch for predators, but only some of these observations have been supported by electrophysiological data [29].

Though some have argued against the universal existence of sleep, no group has provided convincing evidence of a species that does not sleep [28]. Sleep in reptiles and amphibians, including the bullfrog, has proven to be challenging to study, resulting in sparse sleep data on these animals. However, Libourel and Herrel recently reviewed all the available data and concluded that, despite the 1967 bullfrog study, most reptiles and amphibians do fulfill the behavioral criteria of sleep [30]. Sleep-swimming in coral reef fish and unihemispheric sleep in dolphins both provide examples of sleep-like behavior occurring in the absence of total immobility. The evolution of these particular sleep strategies to meet the unique requirements of these species highlights the necessity of sleep. These examples also suggest that our current behavioral criteria for sleep may require adjustments to account for species that engage in specific movements during sleep. While there are examples of species that may not adhere to our current behavioral criteria of sleep, the general consensus among sleep researchers is that all animals studied have exhibited at least some evidence of sleep behavior [28].

Sleep in Drosophila

In 2000, Hendricks [20] and Shaw [21] published independent papers establishing *Drosophila melanogaster* as a viable model system for studying sleep. Hendricks reported that *Drosophila* do indeed assume a specific posture during periods of immobility [20]. Using a locomotor activity monitor, in which single flies are housed in narrow tubes with infrared beams running across them to detect movement, Hendricks and Shaw both determined that flies are immobile for nearly half of their day, and that this immobility occurs mostly at night [20,21]. During these periods of immobility, flies

are less responsive to physical stimuli, indicating an increased arousal threshold [20,21]. Following sleep deprivation by constant mechanical stimulation, *Drosophila* experience a sleep rebound in which they sleep longer than their baseline sleep amount [20,21]. Thus, fruit flies fulfill the behavioral criteria of sleep.

Sleep in the fruit fly has also been shown to have several similarities to sleep in mammals. For instance, sleep in *Drosophila* can be modulated by sleep-effecting drugs in the same manner that these drugs affect mammalian sleep. The adenosine A1 agonist cyclohexyladenosine [20] and the antihistamine hydroxyzine [21], both of which are sleep-inducing in mammals, were shown to increase sleep in flies. Other drugs that have been shown to modulate sleep in the fruit fly include the wake-promoting stimulant Modafinil [31], and the sleep-promoting $GABA_A$ agonist Gaboxadol [32,33]. Furthermore, Shaw demonstrated a gradual decrease in sleep amount over the lifetime of the fly, mirroring the decrease in sleep duration observed as mammals age [21]. Shaw also investigated whether the expression of genes known to be modulated by sleep in rats are also modulated by sleep in the fly. He found that “waking genes” upregulated during spontaneous wake or sleep-deprivation in rats, including the electron transport protein encoding gene *cytochrome oxidase C* and the ER chaperone *BiP*, were also upregulated in awake or sleep-deprived flies.

Following these seminal papers by Hendricks [20] and Shaw [21], there has been a massive surge in sleep research in the fruit fly. There are numerous advantages to using *Drosophila* as a model system: flies can be grown quickly and in great numbers, they are cheap and easy to maintain, and most importantly, they are genetically tractable. Within just the past two decades, several sleep-related genes

have been identified using *Drosophila*, and likewise, a number of genetic tools that allow for the manipulation of sleep in the fruit fly have been developed.

The first forward genetic screen for a short-sleeping *Drosophila* mutant identified the voltage-gated potassium channel gene *Shaker* as a sleep modulating gene [34]. *Shaker* mutants sleep 66% less than wildtype controls and have reduced lifespan [34]. A loss-of-function mutation in *Hyperkinetic*, a regulatory subunit of *Shaker*, also produces a short-sleeping phenotype as well as a learning defect [35]. A separate forward genetic screen identified the short-sleeping mutant *sleepless*, which sleeps 80% less than wildtype controls and also exhibits a shortened lifespan [36]. The *sleepless* gene encodes a membrane-bound protein that has been shown to regulate the *Shaker* channel; it was shown later that *sleepless* also regulates nicotinic acetylcholine receptors (nAChR's) [37]. While the discovery of these mutants supports a role in sleep for potassium channels, which help to reduce neuronal excitability by repolarizing the membrane after an action potential, other short-sleeping mutants have implicated neurotransmitters and even a protein degradation pathway in the regulation of sleep.

The dopamine transporter (DAT) clears excess dopamine, a wake-promoting neurotransmitter, from the synaptic cleft. Thus, it is fitting that a mutation in DAT, named *fumin* or sleepless in Japanese, results in a dramatic decrease in sleep [38]. *fumin* mutants are reported to have impaired sleep rebound and a normal lifespan [38], though I found *fumin* mutants to have a shortened lifespan (Appendix I, Fig. 1.1). It has been shown that the short-sleeping phenotype in *fumin* is due primarily to dopamine signaling in the dorsal Fan-shaped Body (dFB), a sleep-promoting area of the fly brain [39]. A double mutant containing both the *fumin* mutation and a dopamine receptor 1 (DA1)

mutation has normal sleep, but expressing DA1 only in the dFB of these double mutants reduces sleep levels back down to those seen in the *fumin* mutant alone [40], suggesting that dopamine signaling specifically in the dFB promotes wake. Direct activation of the dFB by expressing the sodium bacterial channel construct *NaChBac* or the heat activated calcium channel *TrpA* under a dFB promoter has been shown to induce sleep [39].

Another sleep mutant with altered neurotransmitter signaling is the short-sleeping mutant *redeye*, which carries a loss of function mutation in the nAChR subunit α4 and sleeps 50% less than controls [41]. Acetylcholine signaling is typically considered wake-promoting; nAChR's are cation channels that excite neurons when activated, and in mammals ACh is known to be released when animals are awake [42]. Moreover, the Sleepless protein promotes sleep in part by antagonizing nAChR's to reduce excitability in *Drosophila* [37]. However, since loss of function of nAChRα4 promotes wake in *redeye* mutants, this particular nAChR subunit may be enriched in sleep promoting neurons where it normally functions to promote sleep.

More recently, a short-sleeping mutant named *insomniac* (*inc*) that sleeps 65% less than wildtype was discovered [43]. *inc* is thought to encode a BTB-domain adaptor protein for Cullin3, an E3 ubiquitin ligase that is expressed throughout the whole fly [43]. While short-sleeping *inc* nulls have a shortened lifespan, neuron specific expression of *inc-RNAi* produces a short-sleeping phenotype and a normal lifespan [43,44]. The lifespan difference between *inc* nulls and neuronal *inc-RNAi* flies suggests that *inc* function in the body may be important for overall health, but not for the regulation of sleep. Brain specific *cullin3* (*cul3*) *RNAi* also results in a similar short-sleeping

phenotype [43]. While it is difficult to predict how reduced activity of *inc* or *cul3*, which function mainly in protein degradation, would affect sleep, it has been proposed that Inc/Cul3 proteins may target dopamine receptors for degradation, thereby reducing excitability [44]. This theory is supported by pharmacological evidence showing that the short-sleep phenotype of *inc* is lost when dopamine levels are reduced in *inc* mutants by feeding an inhibitor of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis [44].

Overall, the expansion of sleep research to include *Drosophila* has led to a series of advances in our understanding of sleep regulation on the genetic level, and has resulted in the development of a number of genetic tools and can be used to further this understanding in the future.

The Regulation of Sleep

Circadian Regulation of Sleep

Years before sleep research in fruit flies became mainstream, *Drosophila* genetics were being harnessed to uncover the components of the circadian clock. The circadian clock drives the oscillation of various physiological processes over a 24 hour period, telling our body when it is appropriate to eat, sleep, etc. A key feature of the circadian clock is its endogenous nature—once the clock has been entrained, or set, by external cues such as sunlight, it is able to maintain its rhythm in constant conditions without external cues from the environment. While the existence of sleep in all animals

has been debated, the existence of circadian rhythms in organisms from mammals and invertebrates to bacteria and plants has been widely accepted for decades.

The first circadian gene, *period* (*per*), was discovered by Konopka and Benzer in 1971 through a forward genetic screen in *Drosophila* [45]. The *per* gene interacts with three other genes—*timeless* (*tim*), *clock* (*clk*), and *cycle* (*cyc*)—to comprise the core autoregulatory feedback loop of the circadian clock in fruit flies. In very simplified terms, the clock functions as follows: Clk and Cyc proteins dimerize and enter the nucleus where they drive transcription of a number of circadian regulated genes, including *per* and *tim;* as Per and Tim proteins accumulate, they too dimerize and enter the nucleus, inhibiting Clk and Cyc and thereby blocking their own transcription; Per and Tim proteins are eventually degraded, lifting the inhibition of their transcription and allowing the cycle to begin again. The timing of the process is tightly controlled by a number of other proteins that influence the stability of Per and Tim proteins mainly through phosphorylation [46]. The output of this circadian feedback loop is continuous cycling of countless mRNAs and their protein products, resulting in subsequent cycling of the biological processes carried out by these proteins. One major circadian output that can easily be measured in mammals as well as flies is locomotor activity, which has characteristic peaks and troughs throughout the day.

Importantly, though the core mammalian homologs differ slightly in name and number, the components of the *Drosophila* molecular clock are largely conserved in mammals. Oscillation of *per* and *tim* occurs in nearly every tissue in the body, but these peripheral clocks are synchronized by the central clock located within a small group of pacemaker neurons in *Drosophila* [46], or in a tiny region of the hypothalamus called the

suprachiasmatic nucleus (SCN) in mammals [47]. Synchronization of the peripheral clocks to the central clock is thought to occur by the release of a neuropeptide, Pigment Dispersing Factor (PDF) in flies [48] or Vasoactive Intestinal Polypeptide (VIP) in mammals [49], from a subset of the central clock neurons.

The circadian clock plays an important role in dictating the timing of sleep. In humans, a mutation in *per2*, one of the mammalian homologs of *per*, causes Familial Advanced Sleep Phase Syndrome (FASPS). Sleep duration is not affected in people with FASPS, but sleep onset occurs about 4 hours earlier than average [50]. Less dramatic changes in time of sleep onset are caused my natural polymorphisms in clock genes, which can determine whether a person is an early rising "lark," or a late rising "owl" [51,52]. Furthermore, lesions in the mammalian SCN or disruption of the core clock genes in flies and mammals results in locomotor arrhythmicity, or loss of the characteristic daily movement pattern, when animals are kept in constant conditions (without environmental cues) [20,53,54].

In flies, timed secretion of PDF from a subset of the ventral lateral neurons (LNv) has a wake-promoting effect, resulting in an anticipatory peak of activity just before dawn. Loss-of-function mutations in *pdf* or it's receptor *pdfr* result in increased late night sleep [55], while constitutive activation of a group of LNv causes decreased nighttime sleep [56]. PDF specifically activates a group of dorsal circadian neurons (DN1s) that induce arousal by releasing the wake-promoting diuretic hormone 31 (DH31) [57]. The LNv also express inhibitory $GABA_A$ receptors which, when activated, halt PDF release to promote sleep [55]. Expression of the circadian gene *wide awake* peaks in clock neurons at dusk, triggering upregulation of $GABA_A$ receptors to promote sleep [58].

While the role of the circadian clock in the timing of sleep is clear, there is also evidence that the clock can influence sleep duration and sleep rebound. SCN lesions in primates [59] and mice [60] cause increased sleep duration. Moreover, mutations in clock genes in flies and mice can either increase or decrease sleep duration [61,62] [63]. Following sleep deprivation, *Drosophila per, tim,* and *clk* mutants all show extended sleep rebounds, recovering 100% of sleep lost rather than the 30-40% that is typical of wildtype. *cyc* mutants recover up to 300% of sleep lost, never return to baseline sleep, and start to die from sleep deprivation after 10 hours [64]. Mice with double mutations in the circadian clock genes *cry1* and *cry2* instead have reduced sleep rebound after sleep deprivation [63]. Thus, the circadian clock has a strong influence on the timing of sleep, but likely interacts with other mechanisms that control sleep homeostasis.

Mechanisms of Sleep Homeostasis

Organisms depend on internal systems to maintain homeostasis, or a constant equilibrium within the body, allowing for optimal performance. When the body is forced to stay awake for an extended period of time, homeostatic mechanisms ensure that recovery sleep, characterized by longer duration and/or deeper intensity, will be obtained at the next available opportunity. Because the processes controlling sleep homeostasis are largely unknown, these mechanisms are often referred to collectively as the sleep homeostat. In 1982, Borbely proposed the “two process model of sleep regulation,” in which he suggested that the circadian system and the sleep homeostat

work together to regulate sleep. In his model, sleep pressure builds continuously while an organism is awake and triggers sleep when it reaches its upper limit [65].

Though Borbely originally thought that the circadian clock and sleep homeostat had independent influences on sleep, it is now understood that there is some crosstalk between the two processes. As discussed above, altering clock gene expression can impact sleep duration; additionally, prolonged wakefulness can alter the expression of core clock genes [66]. Interaction between the two processes may allow organisms to stay alert even toward the end of the day, when sleep pressure has accumulated but not yet reached its upper limit [67].

Sleep deprivation experiments, which typically involve continuous physical stimulation to prolong wake, are most commonly used to study sleep homeostasis. In animals whose brain activity can be measured by an EEG, slow wave sleep, characterized by prominent delta waves in the 0.5-4.5 Hz range, is considered the best marker for sleep intensity [10]. As sleep deprivation is extended, a corresponding increase in slow wave sleep is observed in the subsequent sleep rebound, as is an increased arousal threshold in the sleeping animal [68]. Additionally, prolonged wakefulness is correlated with increasing theta activity, or waves in the 4-7 Hz range, which is considered a reliable marker for sleep pressure [69]. However, whether there are molecular markers for sleep pressure, and what these markers tell us about the mechanisms of homeostatic sleep regulation, is less clear.

Several molecules have been identified that increase in abundance as sleep pressure builds. For instance, adenosine accumulates in the basal forebrain and cortex during sleep deprivation, and is then depleted during recovery sleep [70]. Furthermore,

blocking A1 adenosine receptors in the basal forebrain during sleep deprivation prevents recovery sleep from occurring, suggesting that adenosine directly drives sleep rebound [71]. Blocking ATP synthesis to cause energy depletion results in accumulation of extracellular adenosine and an increase in sleep [72]. Thus, it is thought that prolonged wakefulness, which results in increased neuronal firing in both the basal forebrain [73] and the cortex [74], depletes energy, thereby increasing adenosine concentration and driving sleep.

Nitric oxide (NO), an important signaling molecule, also accumulates in the basal forebrain during sleep deprivation and precedes the accumulation of adenosine [75]. Blocking the increase in either NO or adenosine during sleep deprivation prevents a subsequent sleep rebound from occurring [76]. Lesioning of specifically the cholinergic cells in the basal forebrain prevents accumulation of adenosine and NO during sleep deprivation [76]. Thus, it has been proposed that extensive firing of basal forebrain cholinergic cells during prolonged wakefulness induces NO production as a stress signal, causing a subsequent spike in adenosine and promotion of sleep [77]. Indeed, sleep deprivation has been shown to trigger a number of stress responses including activation of inducible nitric oxide synthase (iNOS) to produce NO [78], as well as upregulation of the transcription factor nuclear factor kB (NFkB) [79–81], which plays a major role in the innate immune response.

Immune molecules are also involved in sleep homeostasis. Increased sleep during illness is a major feature of sickness behavior in mammals [82], and bacterial infection in flies also increases sleep [83]. Flies deficient in *Relish,* which encodes an NFkB protein, do not exhibit an increase in sleep following infection, and have reduced

baseline sleep [81], indicating a role for NFkB in sleep regulation. NFkB controls expression of a number cytokines, including Interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα), which have both been shown to increase non-rapid eye movement (NREM) sleep in various species [84,85]. Likewise, blocking the function of either of these cytokines results in a decrease in NREM sleep as well as a decrease in sleep rebound [84,85]. The sleep-inducing effects of these cytokines are likely mediated through complex interactions with other sleep-regulating molecules; for instance, IL-1β promotes the release of NO and adenosine, and also interacts with major neurotransmitters, such as serotonin [86].

Adenosine, NO, and the cytokines IL-1β and TNFα represent just a few of numerous molecules that can reliably influence the sleep state of an animal. While these molecules all regulate sleep, they presumably do so in an indirect manner. The components of the sleep homeostat that directly regulate sleep have yet to be determined, but likely share an intimate link with the underlying function of sleep.

Theories on the Function of Sleep

Synaptic Downscaling

Various sleep deprivation studies in humans and mammals performed over the last century have demonstrated that sleep deprivation impairs learning and memory [87]. Further insight has been provided by work in fruit flies demonstrating that short-sleeping mutants have impaired memory [35], and that genetically inducing sleep improves memory [39] and restores the ability to learn in memory mutants [32].

Additionally, gene expression studies in rats have shown that the wake state induces genes involved in synaptic potentiation, or strengthening of synapses, while the sleep state induces genes involved in synaptic depression, or weakening of synapses [79]. This finding has also been supported by evidence in *Drosophila* of sleep-dependent changes in protein levels of synaptic markers [88]. Thus, a popular theory of sleep function is that sleep is necessary for synaptic downscaling, or weakening of synapses, to maintain synaptic homeostasis [89].

The synaptic homeostasis hypothesis proposes that learning occurs during wake, resulting in a net increase in synaptic strength that in turn requires more energy, space, and cellular materials for the brain to maintain [90]. Thus, synaptic downscaling occurs during sleep, at a time when most synapses are less active, to globally normalize synaptic strength down to a sustainable level [90]. Accordingly, it is proposed that sleep after learning improves memory consolidation because synaptic downscaling increases the signal-to-noise ratio between new memories and old, poorly-integrated ones [91]. Structural evidence in flies supports the theory of synaptic downscaling; synapses in three different neuronal circuits increase in size or number during wake and decrease only following sleep [92]. However, the system may be more complex in mammals—different observations have been made depending on the type of synapse studied and the type of experience preceding sleep [93].

Energy Restoration

A simple and more cellular theory on the function of sleep is that sleep allows for the restoration of depleted energy stores in the brain. Compared to other organs, the

brain has an incredibly high metabolic rate—it makes up only 2% of our body mass, but consumes 20% of available oxygen [94]. However, positron-emission tomography (PET) studies have shown that the human brain consumes only half as much glucose during deep sleep as it does during wake [95–97]. The markedly lower metabolic rate during sleep could provide an opportunity for energy replenishment.

While circulating brain glucose levels don't appear to differ between sleep and wake states [98], some evidence suggests that glycogen stores become depleted during the wake state. A study in flies reported that sleep deprivation resulted in decreased glycogen stores in the brain and body during the first 3 hours [99]. Similar findings were reported in some mammalian studies, but were contradicted by other findings [100–104]. These variable results may be a consequence of glycogen's sensitivity to dissection conditions. ATP is another molecule that could serve as a marker for the energy state of the cell, but is incredibly sensitive to oxidation, making measurement difficult. An alternative approach is to measure phosphorylation of adenosine monophosphate kinase (AMPK), which occurs when ATP is deleted and adenosine monophosphate (AMP) levels are high. Sleep deprivation in mice does result in high levels of phopho-AMPK, suggesting a state of energy depletion [105]. However, more direct methods of measuring energy stores are needed to properly investigate this theory.

Metabolite Clearance

The more recent discovery in mice of a sleep-activated brain glymphatic system, which parallels the lymphatic system of the body, suggests that sleep may serve to

clear harmful metabolites from the brain [106]. Xie et al. reported a 60% increase in interstitial space in the brain during sleep, allowing for increased convective flow between cerebrospinal fluid and interstitial fluid. The group demonstrated that this sleep-dependent increase in convective flow resulted in better clearance of β-amyloid (Aβ) the protein implicated in the pathogenesis of Alzheimer's disease. Poor sleep quality is a predictor of Alzheimer's disease [9], suggesting that inadequate sleep-driven clearance of Aβ may contribute to its aggregation and the development of the disease.

The glymphatic system could also serve to clear reactive oxygen species (ROS), reactive molecules that are produced by incomplete reduction of oxygen during oxidative phosphorylation. ROS can covalently bind to and inhibit the function of proteins, lipids, and DNA, posing a serious threat to the cell. Due to their high metabolic rate and exposure to additional ROS from neurotransmitter metabolism [107], neurons are particularly at risk of oxidative damage from ROS. Since the brain is more metabolically active during the wake state than during sleep, it is possible that one purpose of sleep is to allow for the clearance of ROS from the brain. This theory was first proposed in 1994 by Reimund, who termed it the Free Radical Flux Theory of Sleep [107].

Reimund posited that ROS accumulate in the brain during the wake state, and the lower metabolic rate of sleep provides the brain's antioxidant system with the opportunity to catch up, neutralizing neuronal ROS down to baseline levels in preparation for the next day's cycle. Reimund argued that the Free Radical Flux Theory provides an explanation for the puzzling observation that smaller mammals tend to sleep more and have shorter lifespans. This phenomenon is difficult to explain if the

purpose of sleep is learning and memory. However, if the purpose of sleep is to clear ROS, then smaller mammals, which tend to have higher metabolic rates, would accumulate ROS faster, require more sleep, and potentially die earlier due to the deleterious consequences of oxidative damage.

Reimund's theory was purely hypothetical, but a handful of groups tested his theory, all using variations on the standard disk over water technique in which rodents are placed on a small platform above water in order to prevent sleep for extended periods of time. This technique was reported to cause an increase in amino-cupric-silver staining, a general indicator of cell damage [108] and increased lipid peroxidation, an indicator of oxidative damage [109] in the brains of sleep deprived rodents. Others observed decreased levels of glutathione [110] or decreased SOD1 activity [111] in the brain, as well decreased glutathione levels and catalase activity in the liver [112] of sleep deprived rats. However, other groups published contradictory findings, reporting no change in antioxidant activity and no evidence of oxidative damage in the brains of sleep deprived rats [113–115]. Reimund did not speculate on a role for ROS in the regulation of sleep, but it has been reported that oxidized glutathione, extracted from the brains of sleep deprived rats, induces sleep when injected into control rats [116]. This finding was supported by a later report that injection of a chemical oxidant into the brains of rats also induces sleep [117].

Though flies offer a simple system with strong genetic advantages, few groups have investigated the relationship between ROS and sleep in *Drosophila.* It has been observed that feeding flies a low dose of paraquat, an herbicide that catalyzes ROS production, results in sleep fragmentation occurring earlier than is typically observed in

aged flies [118]. Additionally, inducing sleep fragmentation by light cycle interruption results in higher levels of ROS in middle-aged flies, and in induction of a number of genes that are induced by high levels or ROS [119].

While the relationship between sleep and ROS remains unclear, several genetic and pharmacological tools have yet to be utilized to investigate this relationship in *Drosophila*. In the following chapter, I use a diverse set of short-sleeping *Drosophila* mutants, alongside other genetic and pharmacological methods of sleep manipulation, in order to elucidate the role of ROS in the function and regulation of sleep.

Chapter II: A bi-directional relationship between sleep and oxidative stress

Adapted from a manuscript currently under revision for publication at PLoS Biology.

Vanessa M. Hill[1], Gunter B. Sissoko[2], Ifeoma S. Irobunda[2], Stephen Leong[2], Julie C. Canman[3], and Mimi Shirasu-Hiza[1]

[1] Department of Genetics and Development; Columbia University Medical Center; NY, NY, 10032; USA

[2] Columbia University; NY, NY, 10027; USA

[3] Department of Pathology and Cell Biology; Columbia University Medical Center; NY, NY, 10032; USA

Abbreviations

ROS = reactive oxygen species; nAChRs = nicotinic acetylcholine receptors; DAT = dopamine transporter**;** UAS = upstream activation sequence; Inc = insomniac; Cul3 = Cullin-3; RNAi = RNA interference; dFB = dorsal Fan-shaped Body; SEM = standard error of the mean.

Abstract

Though sleep appears to be broadly conserved in animals, the physiological functions of sleep remain unclear. In this study, we sought to identify a physiological defect common to a diverse group of short-sleeping *Drosophila* mutants, which might provide insight into the function and regulation of sleep. We found that these short-sleeping mutants share a common phenotype of sensitivity to acute oxidative stress, exhibiting shorter survival times than controls. We further showed that increasing sleep in wild-type flies using genetic or pharmacological approaches increases survival after oxidative challenge. Moreover, reducing oxidative stress in the neurons of wild-type flies by overexpression of antioxidant genes reduces the amount of sleep. Together, these results support the hypothesis that a key function of sleep is to defend against oxidative stress and also point to a reciprocal role for ROS in neurons in the regulation of sleep.

Introduction

A sleeping animal is vulnerable to predators and other dangers in its environment for a large portion of the day. Despite these daily risks, sleep is an evolutionarily conserved behavior throughout the animal kingdom [11,28,120], suggesting that sleep serves important functions. In support of this, prolonged episodes of acute sleep deprivation in both rodents and invertebrates cause an increased need to sleep [69,121–123], cognitive impairment [124,125], increased metabolic rate [123,126], and death [64,123,126]. It remains unclear whether these effects are due to loss of sleep or due to the intense stress associated with acute sleep deprivation. Epidemiological studies have revealed that chronic sleep restriction, or shortened sleep duration, in humans is

associated with metabolic disorders [7], cardiovascular disease [8], inflammation [127,128], psychiatric disorders [129], and even premature mortality [130,131]. Similar to experimental results involving acute sleep deprivation, it is unclear whether these defects are due to the loss of sleep itself, to associated disruptions in circadian rhythm, or from the very factors that cause sleep loss, such as shift work, aging, or psychological stress. Thus, while current research in both humans and model organisms has demonstrated an important role for sleep in learning and memory [32,35,39,132], it has been difficult to identify underlying functions for sleep essential to the organism's survival or fitness.

Sleep is thought to be regulated by two distinct types of mechanisms: those that control the timing of sleep, such as the circadian system, and those that control the duration of sleep, also called sleep homeostasis mechanisms [65,133]. While the molecular mechanisms underlying circadian regulation have been well characterized, molecular mechanisms regulating sleep homeostasis are less well-defined, but thought to be neuronally based [37,133–137] and context-dependent—that is, sleep deprivation or other stress conditions may induce different homeostasis pathways than baseline sleep. Because acute sleep deprivation increases sleep need and results in extended sleep duration at the animal's next opportunity to sleep, many models of sleep homeostasis propose a feedback mechanism in which the wake state increases sleep-promoting factors, such as adenosine or overall synaptic strength [133,137]. The sleep state then clears or abrogates these factors to allow the wake state.

A controversial hypothesis for the function of sleep is the free radical flux theory of sleep, proposed in a theoretical paper by Reimund in 1994. Reimund proposed that

reactive oxygen species (ROS) accumulate in neurons during the wake state and that sleep allows for the clearance of ROS in the brain [107]. ROS are chemically reactive by-products of metabolism, which, when not properly neutralized, cause damaging covalent modifications that inhibit the function of proteins, lipids, and DNA and can lead to cell death. Thus, the free radical flux hypothesis proposed that the core function of sleep is to act as an antioxidant for the brain. Despite the appeal of this hypothesis, data to support it are conflicting. While some groups have reported decreased antioxidant capacity and oxidative damage in the brains of sleep-deprived rats and mice [108–111], other reports have contradicted these findings [113,114,138]. As a result, the Reimund hypothesis has fallen out of favor as a model for sleep function. Notably, all studies testing the Reimund hypothesis focused on the effects of acute sleep deprivation. In contrast to acute sleep deprivation, the relationship between chronic sleep restriction and oxidative stress has not been thoroughly investigated, despite the physiological relevance of chronic sleep restriction widespread in modern society [139].

In recent years, the fruit fly has become a powerful, genetically tractable model system for the study of sleep [20,21]. Forward genetic screens have identified a number of *Drosophila* mutants that are short-sleeping and retain intact circadian rhythms. Loss-of-function mutations in ion channels and ion-channel regulators, including *sleepless,* which regulates the potassium channel Shaker and nicotinic acetylcholine receptors (nAChRs), have been shown to reduce sleep [34–37]. Other short sleep causing mutations include the *redeye* allele of the nAChRα4 subunit [41], the *fumin* allele of the dopamine transporter (DAT) [38], and loss of function of the putative ubiquitin ligase adaptor encoded by *insomniac* (*inc*) [43,44]. It has been hypothesized that these

mutations cause short sleep by increasing neuronal excitability [133]. These mutants allow researchers to investigate the effects of chronic sleep restriction independent of circadian defects. Though the specific genes affected vary widely, the common phenotype of these diverse mutants is chronic short sleep. Thus, these mutants provide an ideal system for identifying a "core" or essential function of sleep; if chronic short sleep has negative effects on health, these short-sleeping *Drosophila* mutants should share a common physiological defect.

In this study, we sought to identify a physiological defect common to short-sleeping flies that might provide insight into the function and regulation of sleep. We found that diverse short-sleeping mutants are sensitive to acute oxidative stress, exhibiting shorter survival times than controls, and that increasing total sleep duration of wild-type flies promotes survival after oxidative challenge. We further showed that neuronal overexpression of antioxidant genes in wild-type flies reduces sleep. Our data demonstrate that one function of sleep is to increase the organism's resistance to oxidative stress and support the hypothesis that sleep abrogates neuronal oxidative stress; these results also point to a role for neuronal ROS in the homeostatic regulation of sleep.

Results

Neuronal reduction of *inc* does not compromise lifespan, metabolism or immunity.

To identify specific physiological functions of sleep (Fig. 2.1A), we first focused on

neuron-specific RNAi of the *insomniac* (*inc*) gene, which has been shown to cause short sleep [43,44]. *inc* encodes a putative adaptor protein for Cullin-3 (Cul3), an E3 ubiquitin ligase expressed in both the brain and the body. Cul3 is involved in a number of crucial biological processes, and *inc* null mutants have reduced lifespan [43]. In contrast, neuron-specific RNAi of *inc* was reported to cause short sleep without affecting lifespan [43], suggesting that reduction of Inc activity in non-neuronal tissues affects lifespan in a sleep-independent manner. For this reason, we used flies expressing neuron-specific *inc-RNAi* as our initial model of short sleep.

We verified that animals expressing a *UAS-inc-RNAi* construct via the pan-neuronal driver *elav-GAL4*, hereafter referred to as neuronal *inc-RNAi* flies, exhibited a 30% reduction in total sleep time relative to isogenic controls carrying one copy of either the *inc-RNAi* construct or *elav* driver alone (Fig. 2.1B, $p<0.0001$ relative to either control). We further confirmed that neuronal *inc-RNAi* flies exhibit normal lifespan compared to controls (Fig. 2.1C, $p>0.5$ compared to either control), consistent with a previous report [43] and with recent findings on inbred short-sleeping *Drosophila* lines that have normal lifespan [140]. This result confirms earlier findings that chronic short sleep (equivalent to a person sleeping 5.6 hours instead of 8 hours per night) does not itself shorten lifespan.

Changes in sleep are often associated with altered metabolic energy storage. In humans and mice, sleep loss is associated with metabolic dysfunction such as obesity [141,142] and in flies, prolonged sleep is associated with increased starvation resistance [143]. We tested whether neuronal *inc-RNAi* flies have altered starvation resistance, which reflects altered metabolic energy stores. We found that the mortality

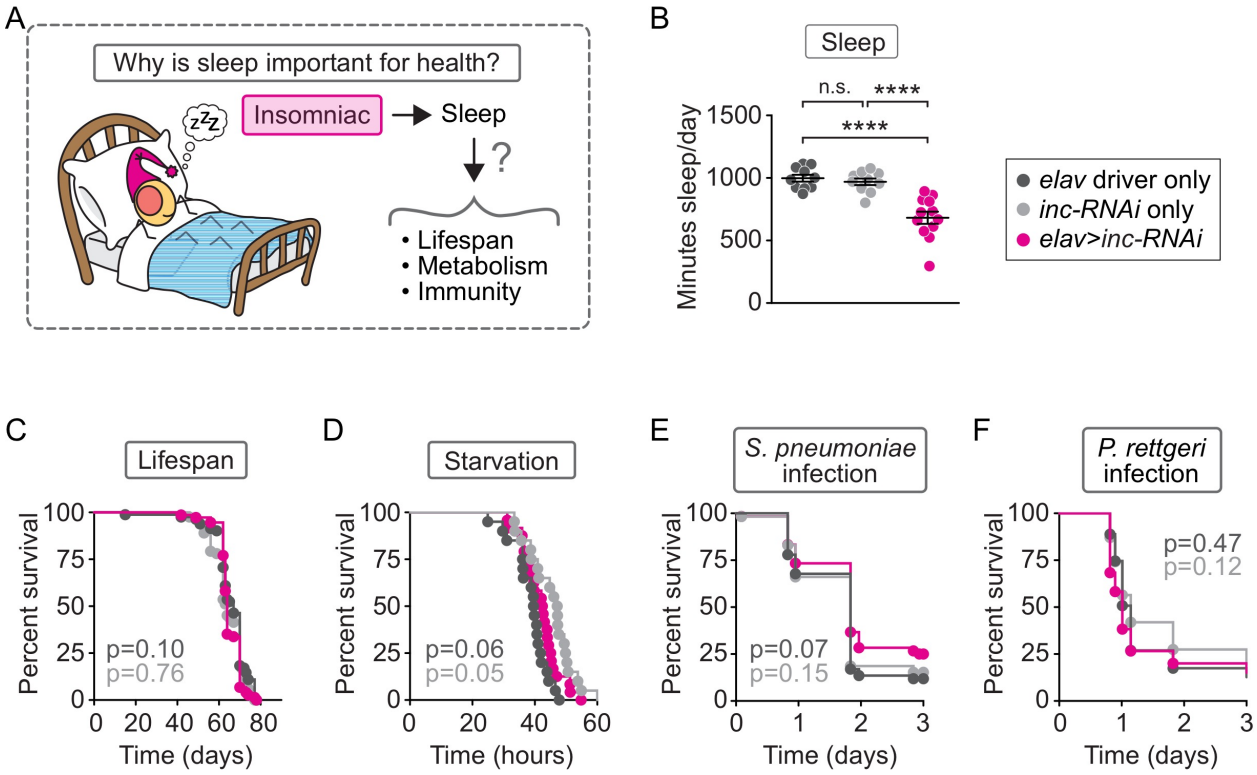

Fig. 2.1. Neuronal *inc-RNAi* reduces sleep without affecting lifespan, metabolism, or immunity. We investigated the importance of sleep in the health of neuronal *inc-RNAi* flies by examining three specific health parameters: lifespan, metabolism, and immunity (A). Relative to genetic controls, neuronal *inc-RNAi* flies sleep 30% less than controls (B, $p<0.0001$ compared to either control, n=10-12 flies/genotype), display a normal lifespan (C, $p>0.05$ compared to either control, n=206-225 flies/genotype), die from starvation at an intermediate rate (D, $p>0.05$ compared to driver control, $p=0.05$ compared to *inc-RNAi* control, n=20-24 flies/genotype), and die at the same rate as controls after injection with *S. pneumoniae* (E, $p>0.05$ compared to either control, n=59-60 flies/genotype) and *P. rettgeri* (F, $p>0.05$ compared to either control, n=60-63 flies/genotype). For the scatter plot in (B), each data point represents the average sleep in minutes/day measured across 5 days for an individual animal. Data are shown as mean ± SEM. p-values were obtained by ordinary one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons (B) or by log-rank analysis (C-F). Data from representative experiments are shown. Lifespans were performed twice. All other experiments were performed at least 3 times.

rate of neuronal *inc-RNAi* flies after starvation was intermediate between normally sleeping controls that express only the *elav* driver or the *UAS-inc-RNAi* construct alone (Fig. 2.1D, p=0.0592 compared to *elav* control, p=0.0493 compared to *inc-RNAi* control), suggesting that short sleep does not affect metabolic energy storage in neuronal *inc-RNAi* animals.

Acute sleep deprivation has also been associated with immune dysfunction in humans, rats, and mice [144–147]. To assay for defects in immunity due to chronic short sleep, we injected neuronal *inc-RNAi* flies with different bacterial pathogens, including *Streptococcus pneumonia*, a Gram-positive pathogen that has been well-characterized in *Drosophila* (Fig. 2.1E), *Providencia rettgeri*, a Gram-negative natural pathogen found in wild-caught *Drosophila* (Fig. 2.1F), *Listeria monocytogenes,* and *Staphylococcus aureus* (Fig. 2.2A-B). In each case, neuronal *inc-RNAi* flies died at the same rate as one or both of their genetic controls. To further test whether chronically reduced sleep causes deficits in immune function, we examined the response of short sleeping *fumin* mutants that lack a functional dopamine transporter [38]. We confirmed earlier findings that *fumin* mutants exhibit short sleep (~95% reduction in sleep relative to controls) (Fig. 2.5C, left panel, p<0.0001). We found that *fumin* mutants responded variably to these pathogens (Fig. 2.2C-F). The lack of a consistent immunity defect across different pathogens in both neuronal *inc-RNAi* flies and *fumin* mutants suggests that chronic short sleep does not have a dramatic or common impact on immune function in *Drosophila*.

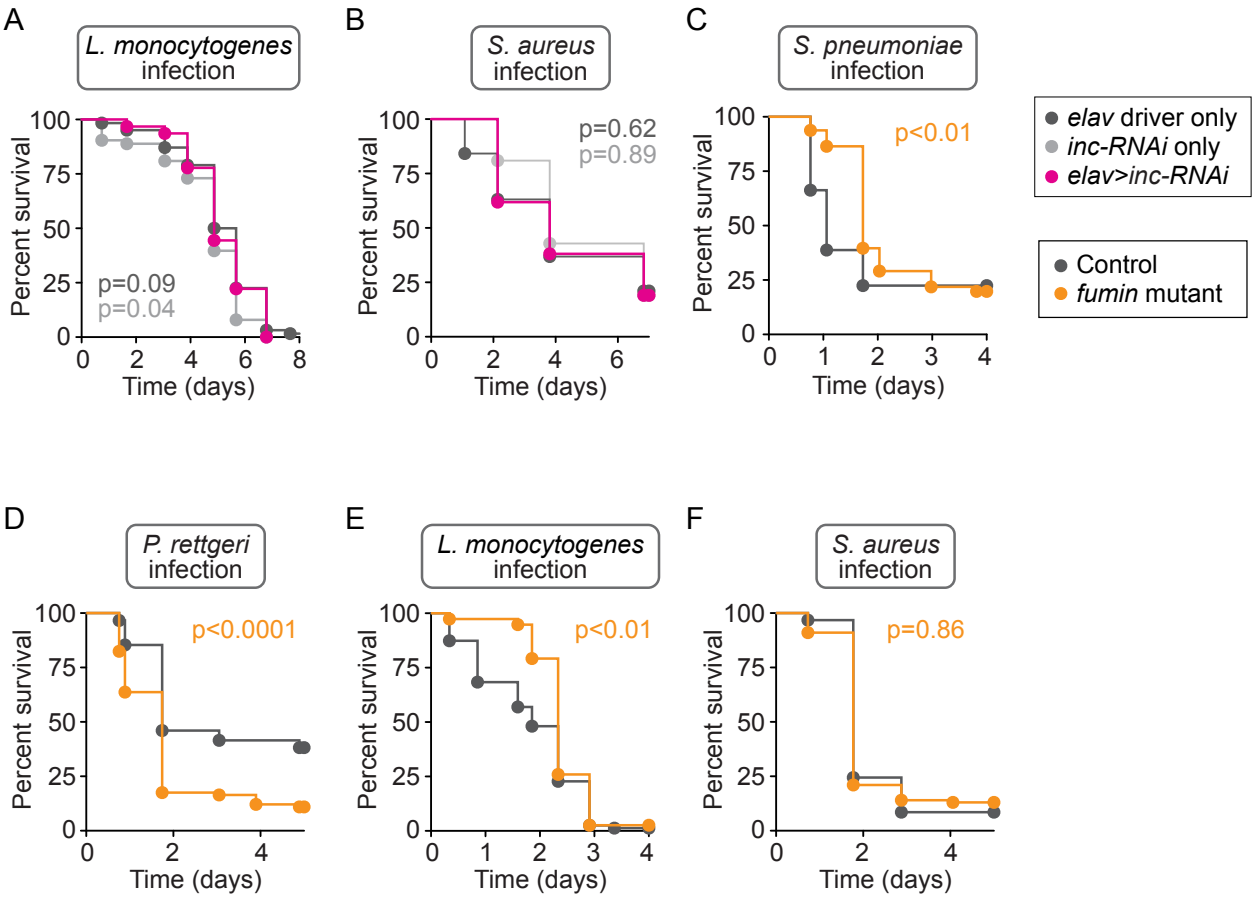

Fig. 2.2. Neuronal *inc-RNAi* flies and *fumin* mutants do not display a global immunity defect. Neuronal *inc-RNAi* flies die at the same or a slightly slower rate than genetic controls after injection with *L. monocytogenes* (A, p=0.09 compared to *elav* control, p=0.04 compared to *inc-RNAi* control, n=62-63 flies/genotype) and die at the same rate as controls after injection with *S. aureus* (B, p>0.05 compared to either control, n=19-21 flies/genotype). *fumin* mutants die slower than controls after injection with *S. pneumoniae* (C, p<0.01, n=96-98 flies/genotype), die faster than controls after injection with *P. rettgeri* (D, p<0.0001, n=89-91 flies/genotype), die slower than controls after injection with *L. monocytogenes* (E, p<0.01, n=77-79 flies/genotype), and die at the same rate as controls after injection with *S. aureus* (F, p>0.05, n=94-100 flies/genotype). p-values were obtained by log-rank analysis. Data from representative experiments are shown. Each experiment was performed at least 3 times.

Short sleep via reduction of *inc* causes sensitivity to oxidative stress

We next set out to test whether sleep is required to defend against oxidative stress [107]. We compared the survival of neuronal *inc-RNAi* flies relative to controls when subjected to two different treatments that induce oxidative stress by increasing ROS levels (Fig. 2.3B). We first injected neuronal *inc-RNAi* flies with a lethal dose of paraquat, an herbicide that catalyzes the production of superoxide anions [148]. We found that neuronal *inc-RNAi* flies died at a significantly faster rate after paraquat injection than controls (Fig. 2.3B, left panel, $p<0.0001$ relative to either control). To determine whether neuronal *inc-RNAi* flies have a specific sensitivity to superoxide anions or if they are also sensitive to other forms of oxidative stress, neuronal *inc-RNAi* flies and controls were fed hydrogen peroxide (H_2O_2), an oxidant that produces highly reactive hydroxyl radicals. Similar to paraquat injection, neuronal *inc-RNAi* flies were sensitive to H_2O_2 feeding compared to controls (Fig. 2.3B, right panel, $p<0.0001$ relative to either control). These results indicate that short-sleeping neuronal *inc-RNAi* flies are susceptible to oxidative stress.

To verify that oxidative stress sensitivity is caused by the reduction in *inc* expression, rather than an off-target effect of RNAi, we next tested *inc* null mutants for paraquat sensitivity. We confirmed that *inc* null mutants exhibit a 50% reduction in sleep (Fig. 2.4A, $p<0.0001$ for both inc^1 and inc^2 mutants relative to controls), as previously reported [43]. Consistent with neuronal *inc-RNAi* flies, *inc* null mutants died faster than controls when injected with paraquat (Fig. 2.3C, $p<0.0001$ for both inc^1 and inc^2 mutants relative to controls). Furthermore, because Inc is a putative adaptor for the Cul3 ubiquitin ligase, we predicted that reduction of neuronal *Cul3* activity would also cause

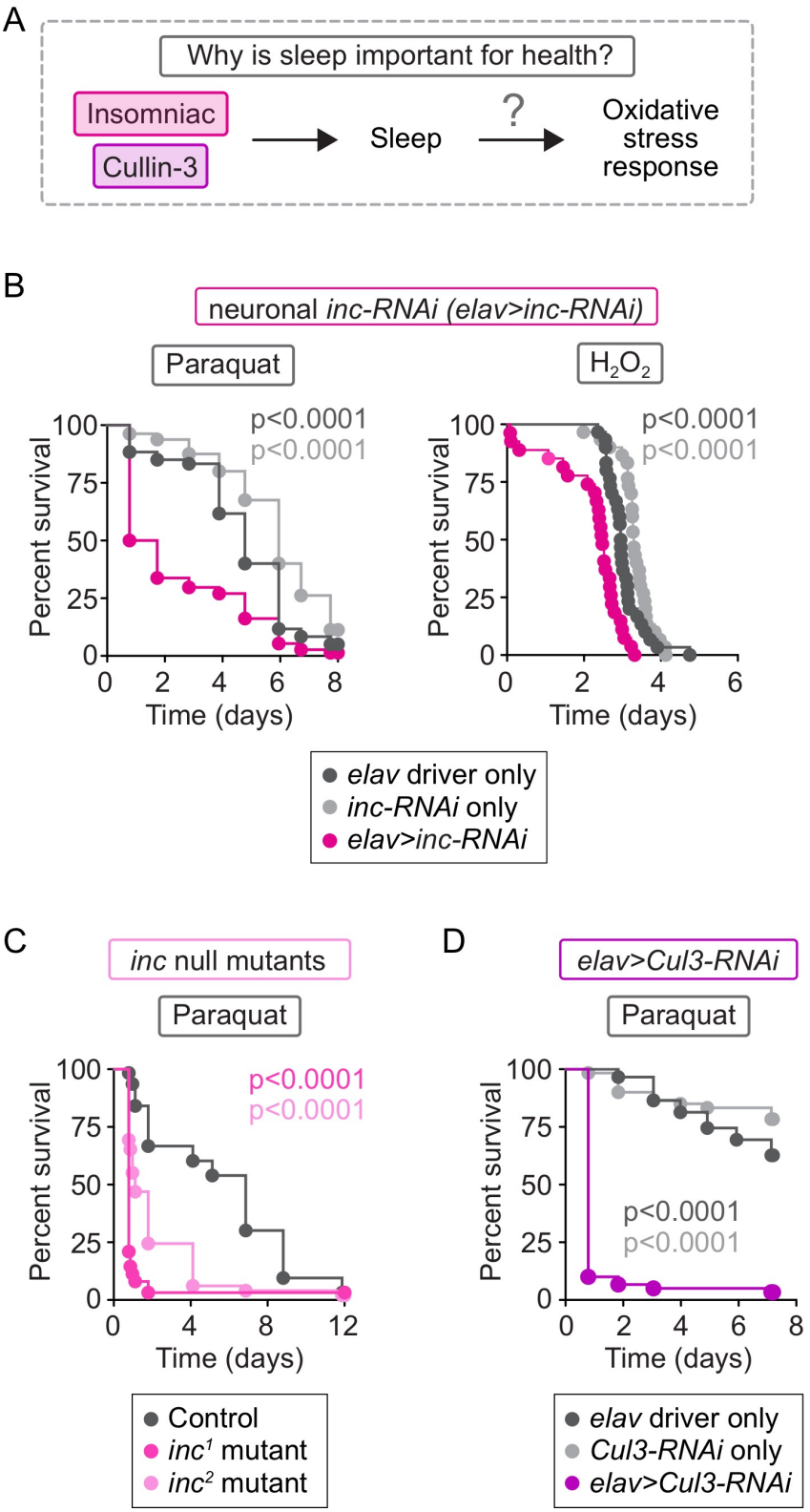

Fig. 2.3. Reducing *inc* or *Cul3* expression results in sensitivity to oxidative stress. We investigated whether reduction of *inc* or *Cul3*, either of which causes short sleep, affects the oxidative stress response (A). Neuronal *inc-RNAi* flies died faster than controls after paraquat injection (B, left panel, $p<0.0001$ compared to either control, n=60-80 flies/genotype) and H_2O_2 feeding (B, right panel, $p<0.0001$ compared to either control, n=27-30 flies/genotype). Similar sensitivity to paraquat was observed in inc^1 and inc^2 null mutants (C, $p<0.0001$ for both mutants compared to control, n=49-63 flies/genotype) and neuronal *Cul3-RNAi* flies (D, $p<0.0001$ compared to either control, n=59-60 flies/genotype). p-values were obtained by log-rank analysis. Data from representative experiments are shown. Each experiment was performed at least 3 times.

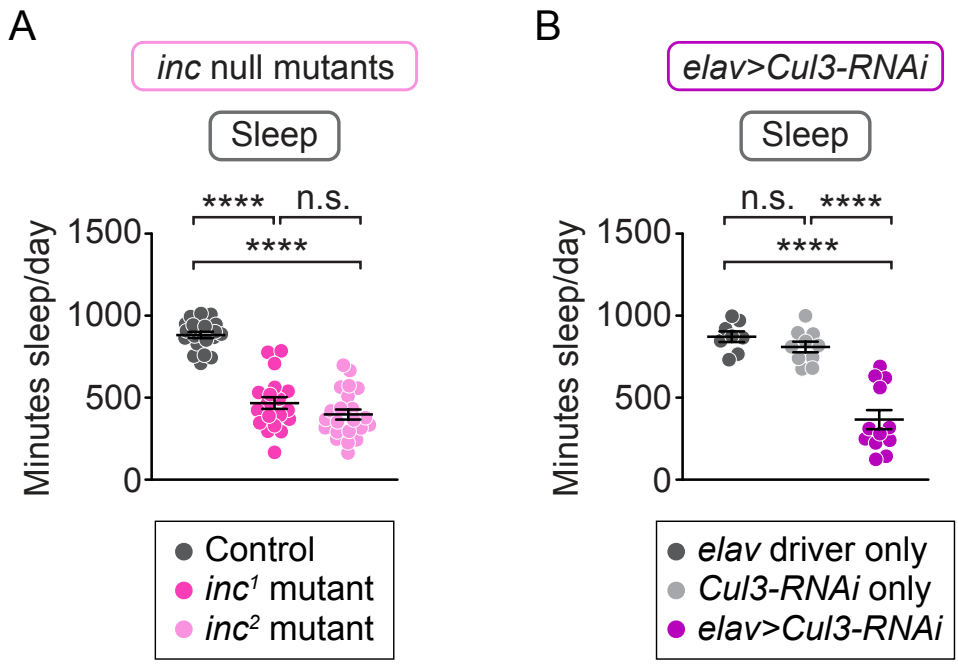

Fig. 2.4. Reduction of *inc or Cul3* causes short sleep.
inc^1 and *inc*^{2} null mutants sleep about 50% than controls (A, p<0.0001 for both mutants, n=20-22 flies/ genotype). *Cul3-RNAi* flies sleep about 60% less than controls (B, p<0.0001 compared to either control, n=40-42 flies/genotype). Each data point represents average sleep in minutes/day measured across 5 days in an individual animal. Data is shown as mean ± SEM. p-values were obtained by unpaired students t-test (A) or by ordinary one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons (B). Data from representative experiments are shown. Each experiment was performed at least 3 times.

paraquat sensitivity. As previously reported [43], neuronal *Cul3-RNAi* flies exhibit a 60% reduction in sleep (Fig 2.4B, $p<0.0001$ relative to either control); here we found that neuronal *Cul3-RNAi* flies were also sensitive to paraquat injection (Fig. 2.3D, $p<0.0001$ relative to either control). Thus, chronic short-sleeping *inc* null mutants and *Cul3-RNAi* flies are sensitive to oxidative stress induced by elevated ROS levels, similar to neuronal *inc-RNAi* flies.

Sensitivity to oxidative stress is common to a diverse group of short-sleeping mutants.

To determine whether sensitivity to oxidative stress is caused specifically by the reduction in *inc* or *Cul3* activity or whether it is more broadly associated with loss of sleep, we next tested for sensitivity to oxidative stress in three different short-sleeping mutants, each carrying mutations in different genes with varied functions: $sleepless^{\Delta 40}$ (*sleepless*), DAT^{fumin} (*fumin*), and $nAChR\alpha4^{rye}$ (*redeye*) (Fig. 2.5A). We first confirmed, as previously reported [36,38,41], that each mutant spends significantly less time sleeping than its isogenic control (Fig. 2.5B-D, left panels, $p<0.0001$ for each). We next tested these short-sleeping mutants for sensitivity to oxidative stress. Relative to controls, we found that each mutant was sensitive to both paraquat injection (Fig. 2.5B-D, middle panels, $p<0.0001$ for each) and H_2O_2 feeding (Fig. 2.5B-D, right panels, $p<0.0001$ for each). Thus, our finding that this molecularly diverse set of short-sleeping mutants has a common susceptibility to oxidative challenge raises the possibility that sleep itself is required for proper response to oxidative stress.

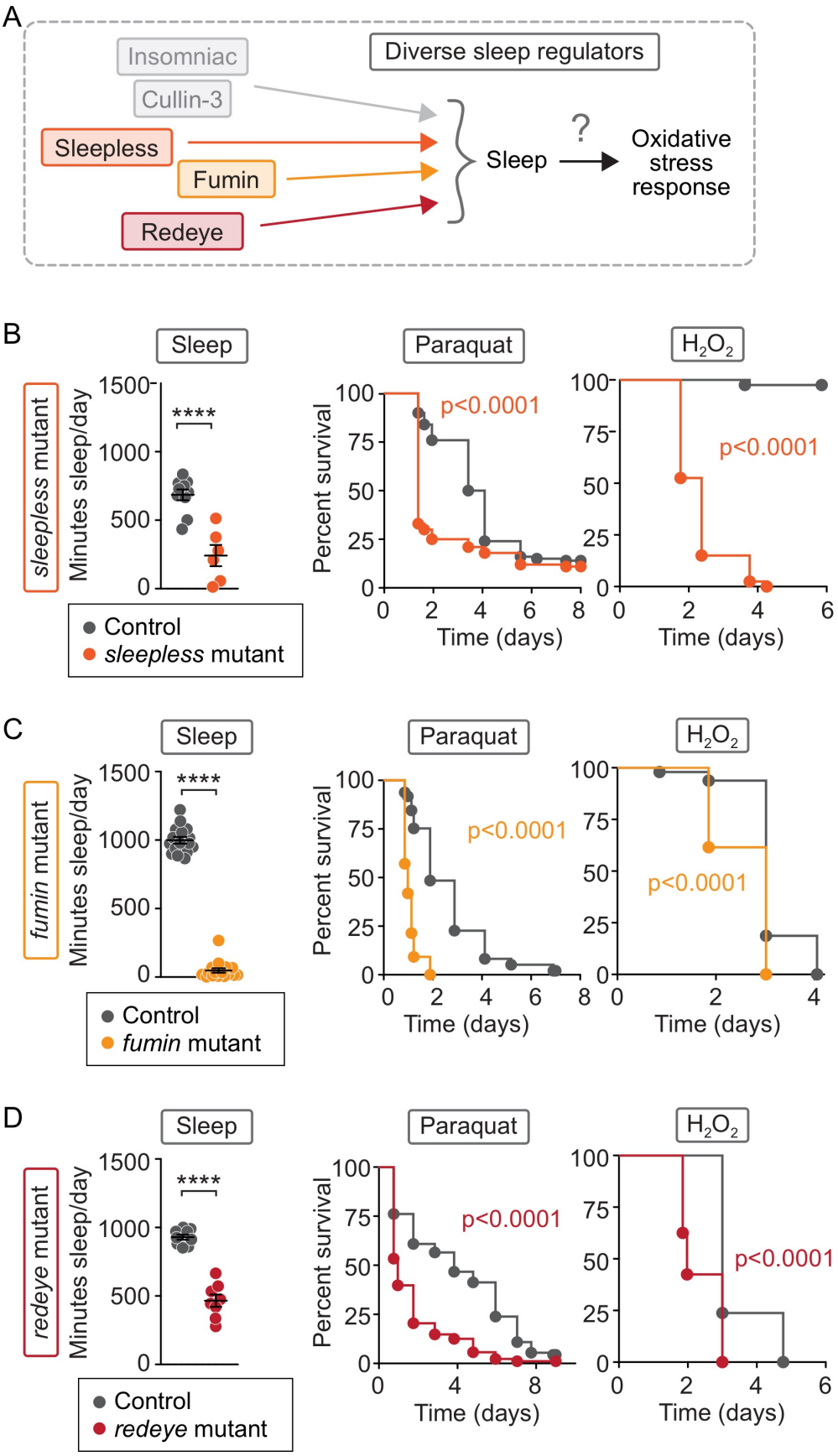
A
Insomniac
Cullin-3
Sleepless
Fumin
Redeye
Diverse sleep regulators
Sleep
?
Oxidative stress response
B
Sleep
Paraquat
H2O2
sleepless mutant
Minutes sleep/day
1500
1000
500
0
****
Control
sleepless mutant
Percent survival
100
75
50
25
0
p<0.0001
p<0.0001
0 2 4 6 8
0 2 4 6
Time (days)
C
Sleep
Paraquat
H2O2
fumin mutant
Minutes sleep/day
****
Control
fumin mutant
p<0.0001
p<0.0001
Time (days)
D
Sleep
Paraquat
H2O2
redeye mutant
Minutes sleep/day
****
Control
redeye mutant
p<0.0001
p<0.0001
Time (days)

Fig. 2.5. A diverse group of short-sleeping mutants is sensitive to oxidative stress.
We asked whether other sleep mutants unrelated to *inc* or *Cul3* share the same sensitivity to oxidative stress (A). *sleepless* mutants sleep 65% less than controls (B, left panel, $p<0.0001$, n=6-10 flies/genotype), *fumin* mutants sleep 95% less than controls (C, left panel, $p<0.0001$, n=16 flies/genotype), and *redeye* mutants sleep 50% less than controls (D, left panel, $p<0.0001$, n=8-9 flies/genotype). When injected with paraquat, *sleepless* (B, middle panel, $p<0.0001$, n=100 flies/genotype), *fumin* (C, middle panel, $p<0.0001$, n=97-98 flies/genotype), and *redeye* (D, middle panel, $p<0.0001$, n=88-92 flies/genotype) mutants all die faster than controls. Faster death relative to controls is also observed by H_2O_2 feeding in *sleepless* (B, right panel, $p<0.0001$, n=40 flies/genotype), *fumin* (C, right panel, $p<0.0001$, n=39-40 flies/genotype), and *redeye* (D, right panel, $p<0.0001$, n=39-42 flies/genotype) mutants. For scatter plots (B-D), each data point represents the average sleep in minutes/day measured across 5 days for an individual animal. Data are shown as mean ± SEM. p-values were obtained by unpaired students t-test (left-panels), or by log-rank analysis (middle and right panels). Data from representative experiments are shown. Each experiment was performed at least 3 times.

Increasing sleep confers resistance to oxidative stress.

Because short-sleeping mutants exhibit sensitivity to oxidative stress, we next tested whether extending sleep duration promotes resistance to oxidative stress. We increased sleep by either genetic manipulation or pharmacological treatment and measured the effect on survival after oxidative challenge. For the genetic approach, we used transgenic flies in which sleep-inducing neurons were activated by the expression of a neuron-activating bacterial sodium channel [39]. For the pharmacological approach, we treated wild-type animals with the sleep-inducing drug Gaboxadol [32,33].

It was previously shown that total sleep time is increased by constitutively activating neurons in the dorsal Fan-shaped Body (dFB), a sleep-promoting region in the fly brain [39]. We verified this phenotype using a previously established dFB driver (*23E10-GAL4*) to drive expression of the neuron-activating bacterial sodium channel construct *UAS-NaChBac* and observed a 40% increase in sleep duration in *dFB>NaChBac* flies (Fig. 2.6A, left panel, $p<0.0001$ relative to either control). We then subjected *dFB>NaChBac* flies to oxidative stress by either paraquat injection or H_2O_2 feeding. In both cases, dFB-activated flies died at a slower rate than controls (Fig. 2.6A, middle and right panels, $p<0.001$ for each). Thus, genetically activating the dFB to increase sleep promotes resistance to oxidative stress.

To further test whether extended sleep duration can increase survival of acute oxidative stress, we used an independent pharmacological method of sleep induction. Wild-type animals were fed the $GABA_A$ receptor agonist Gaboxadol, which induces sleep in *Drosophila* [32,33]. We observed a 25% increase in total sleep time in Gaboxadol-treated animals (Fig. 2.6B, left panel, $p<0.001$) and a corresponding

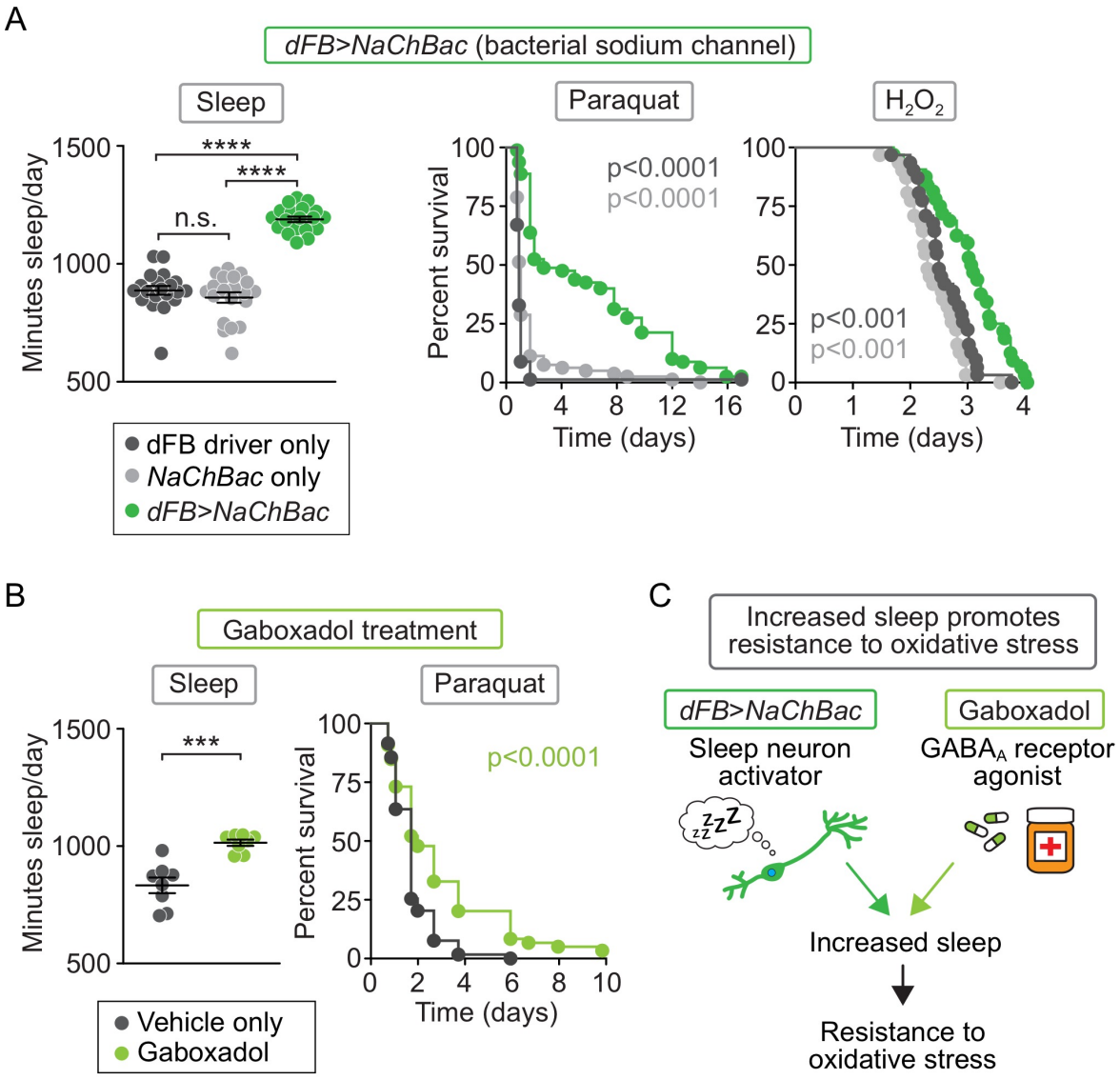

Fig. 2.6. Inducing sleep increases resistance to oxidative stress.
dFB>NaChBac flies sleep 40% more than controls (A, left panel, p<0.0001 compared to either control, n=20 flies/genotype) and die slower than controls after paraquat injection (A, middle panel, p<0.0001 compared to either control, n=79-80 flies/genotype) or H_2O_2 feeding (A, right panel, p<0.001 compared to either control, n=31-32 flies/genotype). Flies fed the $GABA_A$ agonist Gaboxadol sleep 25% more than controls (B, left panel, p<0.001, n=8 flies/condition) and die slower than controls after paraquat injection (B, right panel, p<0.0001, n=118-119 flies/condition). These data support the conclusion that inducing sleep by either genetic or pharmacological means confers oxidative stress resistance (C). For scatter plots (A-B, left panels): each data point represents average sleep in minutes/day measured across 5 days in an individual animal; data are shown as mean ± SEM. p-values were obtained by ordinary one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons (A-B, left panels) or by log-rank analysis (A-B, middle and right panels). Data from representative experiments are shown. Each experiment was performed at least 3 times.

increase in resistance to paraquat injection relative to vehicle-fed controls (Fig. 2.6B, right panel, $p<0.0001$). Together, these results demonstrate that two different methods of increasing sleep both promote resistance to oxidative stress, consistent with the idea that oxidative stress resistance is a physiological function of sleep (Fig. 2.6C).

Neuronal reduction of *inc* causes altered expression of stress response genes.

If sleep clears ROS from neurons, one would expect short-sleeping flies to exhibit higher baseline levels of ROS in the brain. Quantitation of ROS in live brains is extremely difficult, possibly due to tight feedback control of ROS levels via the induction of antioxidant gene expression. As an indirect measure of ROS, we measured the expression of genes known to be activated by high levels of ROS by performing qRT-PCR on the heads of neuronal *inc-RNAi* flies and controls. These genes include the antioxidant genes *SOD1*, *GSTS1*, *GSTO1*, and *catalase*; the mitochondrial stress response genes *hsp60*, *ClpX*, and *Pink1*; and the ER stress response gene *BiP*, which was previously shown to be induced by sleep deprivation [21,149–151]. We found that neuronal *inc-RNAi* flies exhibited increased expression of all of these genes except *catalase* and *BiP* (Fig. 2.7B-I). While neuronal *inc-RNAi* flies had modestly elevated *BiP* expression in the head (Fig. 2.7I), the difference was not significant. Thus, the increased baseline expression of antioxidant genes and mitochondrial stress genes in neuronal *inc-RNAi* flies is consistent with short sleep causing increased ROS levels in the brain.

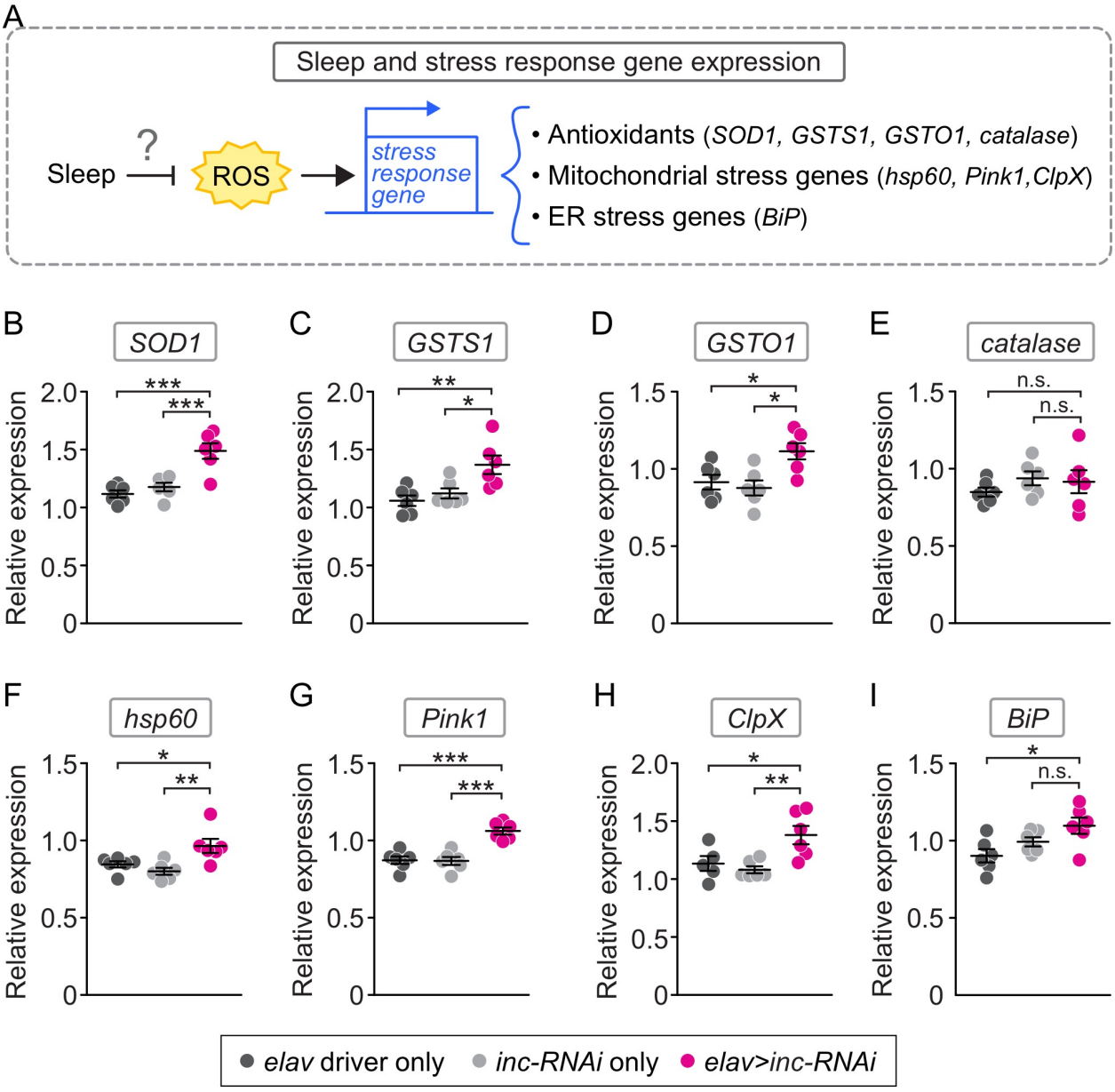

Fig. 2.7. Neuronal *inc-RNAi* heads have increased expression of stress response genes. We investigated whether short sleep affects the expression of three main groups of stress response genes: antioxidant genes, mitochondrial stress genes, and one ER stress gene (A). Neuronal *inc-RNAi* flies have increased baseline head expression of antioxidant genes *SOD1* (B, $p<0.001$ compared either control, n=6 biological replicates per genotype), *GSTS1* (C, $p<0.05$ compared to either control, n=6 biological replicates per genotype), and *GSTO1* (D, $p<0.05$ compared to either control, n=6 biological replicates per genotype), but normal expression of *catalase* (E, $p>0.05$ compared to either control, n=6 biological replicates per genotype). Neuronal *inc-RNAi* flies also have increased basal head expression of mitochondrial stress genes *hsp60* (F, $p<0.05$ compared to either control, n=6 biological replicates per genotype), *Pink1* (G, $p<0.001$ compared to either control, n=6 biological replicates per genotype), and *ClpX*

(H, $p<0.05$ compared to either control, n=5-6 biological replicates per genotype). The ER chaperone gene *BiP* was elevated compared to one, but not both, controls ($p<0.05$ compared to *elav* control, $p>0.05$ compared to *inc-RNAi* control, n=6 biological replicates per genotype). Expression is normalized to *actin*. Data are shown as mean ± SEM. Each data point represents an independent biological replicate with 15-20 individual fly heads per biological replicate. p-values were obtained by ordinary one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons.

Overexpression of antioxidant genes in neurons reduces sleep.

If one function of sleep is to clear ROS from the brain, then it is plausible that ROS itself may be one factor that triggers sleep, perhaps when it reaches a certain critical threshold. To determine whether neuronal ROS levels play a role in the regulation of sleep, we reduced ROS levels in the brains of otherwise wild-type flies by driving neuronal overexpression of the antioxidant genes *catalase*, *SOD1*, or *SOD2* using the *elav-Gal4* driver (Fig. 2.8A). *SOD1* or *SOD2* overexpression resulted in a significant reduction in the total amount of sleep, with an average decrease in total sleep of 10% and 16% respectively (Fig. 2.8B, $p<0.05$ compared to either control). *catalase* overexpression resulted in a similar trend, but did not reach significance compared to the driver control (Fig. 2.8B). Our observation that reducing neuronal ROS levels reduces sleep amount suggests that ROS levels reflect sleep need and play a role in the regulation of sleep.

Discussion

Though sleep appears to be evolutionarily conserved across all animal species [11,28,120], the physiological function of sleep remains unclear. Our data show that chronic sleep restriction sensitizes flies to two types of oxidative stress: paraquat injection and hydrogen peroxide feeding (Fig. 2.3-2.5). Conversely, increasing sleep through either genetic or pharmacological methods promotes resistance to oxidative stress (Fig. 2.6). Thus, our data suggest that one important function of sleep is defense against oxidative stress. The molecular mechanisms underlying the susceptibility of short-sleeping mutants to acute oxidative stress and whether this susceptibility is due to

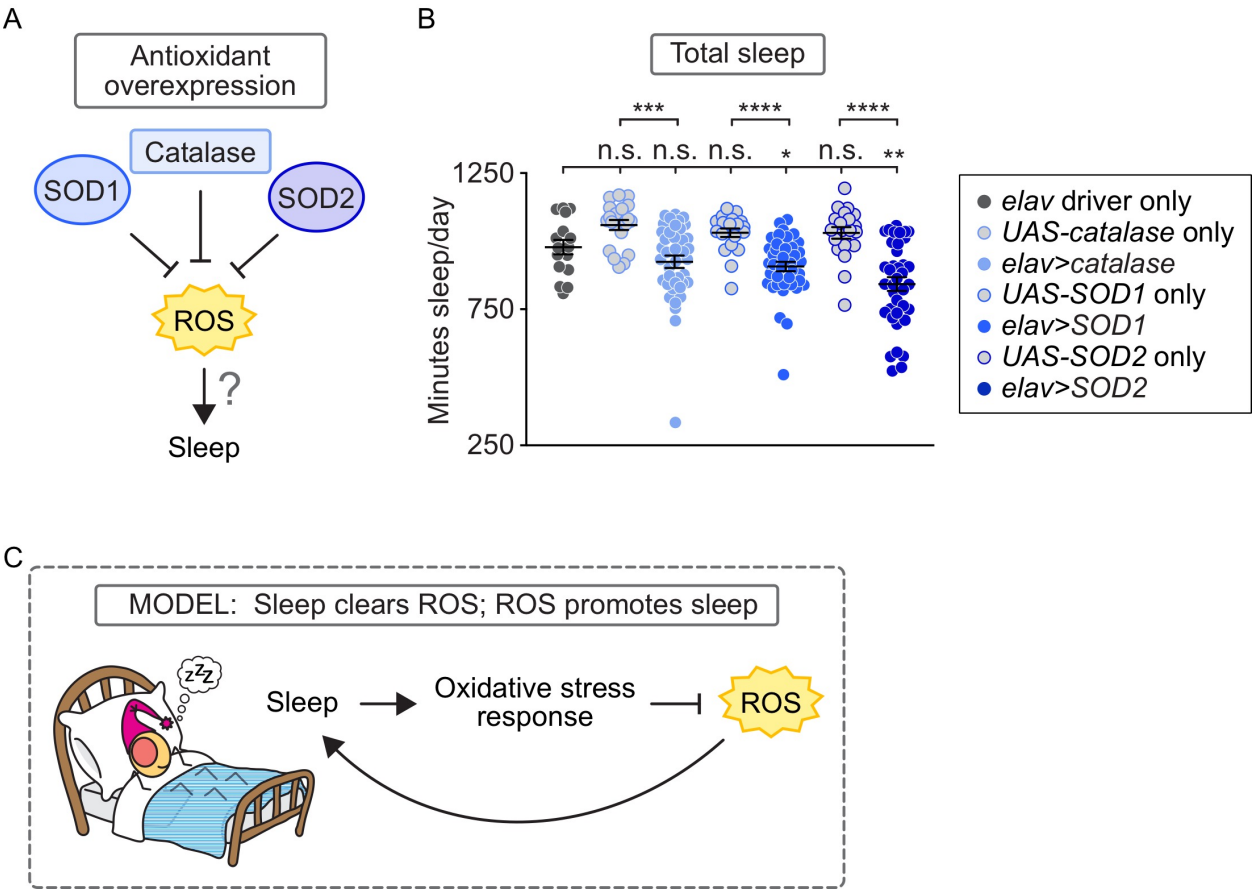

Fig. 2.8. Neuronal overexpression of antioxidants reduces sleep, suggesting a role for ROS in sleep regulation. (A) Neuronal overexpression of the antioxidant genes *SOD1* and *SOD2* reduced sleep by 10% (B, $p<0.05$ compared to either control, n=16-40 flies/genotype) and 16% ($p<0.01$ compared to either control, n=16-38 flies/genotype) respectively. Neuronal overexpression of catalase also reduced sleep, but the decrease was not statistically significant compared to the driver control ($p>0.05$ compared to *elav* control, $p<0.001$ compared to *catalase* control, n=16-40 flies/genotype). Each data point represents average sleep in minutes/day measured across 5 days in an individual animal; data are shown as mean ± SEM. p-values were obtained by ordinary one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons. Pooled data from 2 independent experiments are shown. (B) Model: high ROS levels promote sleep, which in turn clears ROS to promote wake.

the effects of oxidative stress on the brain or other, non-neuronal tissues of the body remains unclear. It is possible that increased baseline ROS levels in neurons or other tissues sensitize short sleepers to acute oxidative stress. Other investigators have found that accumulation of cellular ROS was associated with susceptibility to acute oxidative challenge [152,153]. Chronic sleep loss may lead to accumulated mitochondrial damage that, in the presence of an acute oxidative stress, triggers cell death pathways. Another possibility is that short sleepers are less able to detect or respond to acute oxidative challenge in specific tissues. Testing these hypotheses will be an important focus for future investigation.

Our data also suggest that short-sleeping animals accumulate higher baseline ROS levels in the brain. While ROS levels in the brain are difficult to measure directly, we observed increased expression of antioxidant and mitochondrial stress response genes in the heads of short-sleeping neuronal *inc-RNAi* flies, consistent with increased ROS levels in the brain. Other studies have similarly observed that sleep-deprived animals display increased expression of genes induced by high ROS levels. Induction of the antioxidant regulator *cnc* was observed in fly heads when flies were exposed to recurrent sleep fragmentation [119], and its mammalian homolog *nrf2* was reported to be induced in the cerebral cortex of mice after 6 hours of sleep deprivation [105]. Sleep deprivation has also been associated with activation of the unfolded protein response in the ER in fly heads and mouse brains [21,149–151]. Since both the ER- and mitochondrial unfolded protein responses can be induced by high levels of ROS, we hypothesize that both genetic and environmental sleep loss increase baseline ROS levels that, depending on the specific method of sleep deprivation, genetic background,

and tissue tested, are reflected in the activation of different response pathways.

Finally, we found that increasing antioxidant gene expression in the brain causes short sleep, suggesting that decreasing neuronal ROS levels will promote the wake state. Emerging evidence demonstrates that ROS can act as crucial signaling molecules in a number of biological processes [154,155] and it has been demonstrated that injecting an oxidant into the rat brain induces sleep [117]. Thus ROS levels, either directly or indirectly through the activation of oxidative stress responses, appear to induce sleep.

Taken together, our results support a model for a bi-directional relationship between sleep and oxidative stress in which one function of sleep is to act as an antioxidant for both the body and the brain, increasing the organism's resistance to acute oxidative challenge and reducing ROS levels in the brain; moreover, neuronal ROS plays a role in the regulation of sleep and wake states (Fig. 2.8C). Thus, with chronic sleep restriction, the animal accumulates higher ROS levels in the brain and is sensitive to acute oxidative stress.

Identifying the physiological functions of sleep and key regulators of sleep is critical to understanding the negative effects on health associated with chronic sleep restriction. In the U.S., average sleep time is steadily decreasing [5] and one third of adults sleep less than the recommended 7 hours per night [139]. Sleep restriction is correlated with a variety of diseases [7,8], many of which are also associated with oxidative stress [156–160]. Sleep disturbances have been implicated as a predictor for Alzheimer's, Parkinson's, and Huntington's disease [9,161–163], and in all of these diseases oxidative damage has been reported in the brains of patients postmortem

[164–166]. Because oxidative stress can induce protein misfolding and aggregation through protein damage, neuronal accumulation of ROS is a plausible contributing factor in the pathogenesis of neurodegenerative diseases. Thus, understanding the role of sleep in defense against oxidative stress and the role of ROS in regulating sleep could provide much-needed insight into the pathology and treatment of neurodegenerative diseases.

Materials and Methods:

Fly strains

The following flies were obtained from Nicholas Stavropoulos (New York University): *UAS-inc-RNAi* (VDRC stock #18225), *elav^{C155}-Gal4, inc*^{1} deletion mutant, and *inc*^{2} transposon insertion mutant (CG32810^{f00285}), all in the same genetic background (w^{1118} iso31 or Bloomington stock #5905), along with the isogenic iso31 strain used for outcrossing. In addition, Nick Stavropoulos provided us with *UAS-Cul3-RNAi* (NIG stock #11861R-2) in the NIG *w^{1118}* background along with isogenic control. Parental controls used for experiments were obtained by crossing expression driver (*elav-Gal4*) and RNAi construct (*UAS-inc-RNAi*) lines to the outcrossed wild-type line (iso31) for heterozygous controls, accounting for differences in complex phenotypes affected by genetic background. In case the absence of the *white* gene, which encodes an ABC transporter, has an effect on survival after paraquat or H_2O_2 exposure, red-eyed controls were used with the red- and orange-eyed *inc*^{1} and *inc*^{2} mutants; these *w*$^{+}$ controls were generated by outcrossing *w*$^{+}$ from an Oregon-R background for 8 generations with the *iso31* stock

(Bloomington stock #5905).

redeye, *sleepless*$^{\Delta 40}$ (imprecise excision mutants), and their corresponding background-matched controls were obtained from Amita Sehgal (University of Pennsylvania). *sleepless*$^{\Delta 40}$ was used instead of *sleepless*^{P1} because *sleepless*^{P1} flies were sensitive to wounding, which made paraquat injection experiments difficult to interpret. Male *sleepless*$^{\Delta 40}$ flies also exhibited some wounding sensitivity, whereas females did not, so female *sleepless*$^{\Delta 40}$ flies were used in the paraquat injection experiments. Male *sleepless*$^{\Delta 40}$ were used in H_2O_2 feeding experiments. *fumin* mutants and their background-matched controls were obtained from Rob Jackson (Tufts University).

UAS-NaChBac was obtained from Paul Shaw (Washington University, St. Louis) and *23E10-Gal4* was obtained from Jeffrey Donlea (University of Oxford); both were outcrossed for 8 generations with the iso31 stock. As described above, parental controls used for experiments were obtained by crossing expression driver (*23E10-Gal4*) and transgene construct (*UAS-NaChBac*) lines to the outcrossed wild-type line (iso31) for heterozygous controls.

The following stocks were obtained from the Bloomington Stock Center and outcrossed 6-8 generations into the iso31 background: *UAS-SOD1* (#24754), *UAS-SOD2* (#24492), and *UAS-cat* (#24621).

All flies were raised at room temperature on standard molasses food, and kept on cornmeal food post-eclosion in a temperature controlled (25°C) incubator with a 12-hr light-dark cycle. 5- to 10-day-old males were used for all experiments, unless otherwise noted.

Sleep Analysis and Starvation Assay

Individual flies were loaded into plastic tubes containing cornmeal food, and allowed to acclimate for 1 day. Sleep was monitored for 5 days using Drosophila Activity Monitors (either DAM2 or DAM5) (Trikinetics). Activity was recorded as beam-breaks in 1 min bins and analyzed using PySolo software, with sleep defined as a 5-minute period of inactivity. Graphing and statistical analysis was performed using GraphPad Prism. When comparing two groups: an unpaired t test was performed when standard deviations were similar, and an unpaired t test with Welch's correction was performed when standard deviations were not similar. When comparing three groups, a one-way ANOVA was performed and followed by a Tukey post hoc test to correct for multiple comparisons.

For starvation assays, flies were transferred to tubes containing 1% agar and loaded into *Drosophila* Activity Monitors. Time of death was determined by complete loss of movement.

Lifespan

Flies were collected on the day of eclosion and allowed to mate overnight. Total flies per genotype ranged from 206-225. Males were separated into groups of 20 per vial. Flies were transferred to new vials every 2-4 days and scored for death at time of transfer. Lifespan experiments were performed in two independent trials.

Bacterial and Paraquat Injections

Injections were carried out with a pulled glass capillary needle. A custom-made microinjector (Tritech) was used to inject 50 nL of liquid into the abdomen of each fly. Volume was calibrated by measuring the diameter of the expelled drop under oil. Death was assayed visually at least daily, with a typical n=60 for both bacterial infections and paraquat injections. For each experiment, a smaller set of flies was injected with vehicle alone to ensure that wounding caused minimal death.

The following bacterial strains were used for injections: *Streptococcus pneumoniae* (strain SP1, a streptomycin-resistant variant of D39) obtained from Elizabeth Joyce (University of California, San Francisco) was grown standing in BHI (Brain Heart Infusion media) at 37°C with 5% CO_2, frozen into aliquots with 10% glycerol, pelleted and re-suspended upon thawing, and injected at an OD_{600} of 0.015-0.05; *Providencia rettgeri* (strain Dmel, a natural pathogen isolated from wild-caught *D. melanogaster*) obtained from Brian Lazzaro (Cornell University), was grown shaking in LB at 37°C and injected at an OD_{600} of 0.003-0.005; *Listeria monocytogenes* (strain 10403S) obtained from Julie Theriot (Stanford University) was grown standing in BHI at 37°C and injected at an OD_{600} of 0.075-0.2; and *Staphylococcus aureus* strain 12600 (ATCC), was grown shaking in BHI at 37°C and injected at an OD_{600} of 0.0001-0.001. Post-injection, flies were kept in a 29°C incubator for the remainder of the experiment to allow for optimal infection, with the exception of *P. rettgeri* injection, in which case optimal infection was achieved at 25°C.

For paraquat injections, paraquat (methyl viologen hydrate, Fisher Scientific) was dissolved in water to a concentration of 3–5 mM. Paraquat solution was either stored at

4°C for up to one month, or frozen in aliquots and thawed as needed.

H_2O_2 Feeding Assays

These assays were performed in two ways. In one method, flies were transferred to vials containing a folded Kimwipe soaked with 1.5 mL of a 5% sucrose, 1-4% H_2O_2 solution; flies were flipped onto a freshly soaked Kimwipe every 2 days and death was assayed visually and recorded daily. This method allows very rapid setup (typical experiment used 40 flies/genotype) but provides relatively low-resolution survival kinetics. In the second method, flies were transferred to 5 mm tubes containing a piece of a soaked Kimwipe and loaded into *Drosophila* Activity Monitors, in which case death was determined by a complete loss of movement. Control flies were kept on 5% sucrose alone to ensure that death did not occur by starvation or desiccation. This method provides high-resolution survival kinetics but requires more time-intensive setup (typical experiment used 30 flies/genotype). We found that all our results for short-sleeping mutants were consistent between the two methods.

Survival Curves

Survival curves for starvation assays, lifespan experiments, bacterial infections, paraquat injections, and H_2O_2 feeding assays are all plotted as Kaplan-Meier graphs. Log-rank analysis was performed using GraphPad Prism. All experiments were performed with a minimum of three independent trials (except lifespans, which were done twice) and yielded statistically similar results, except where noted. Graphs and p-values in figures are representative trials.

qRT-PCR

Flies were anesthetized on ice and decapitated. 15-20 heads per sample were homogenized in TRIzol (Invitrogen), and a phenol-chloroform extraction was performed to isolate nucleic acids. Samples were treated with DNAse (Invitrogen) to isolate RNA, and then diluted to a concentration of ~60 ng/uL. RevertAid First Strand cDNA synthesis kit (ThermoFisher) was used to convert RNA to cDNA. Quantitative RT-PCR was performed using a Bio-Rad CFX Connect Real-Time qPCR machine, with Express Sybr GreenER qPCR SuperMix (Invitrogen) and the following primer sets:

SOD1:

For: GGAGTCGGTGATGTTGACCT

Rev: GGAGTCGGTGATGTTGACCT

GSTS1:

For: CACCAGAGCATTTCGATGGCT

Rev: ACGACTGCAATTTTTAGACGGA

GSTO1:

For: ACGACTGCAATTTTTAGACGGA

Rev: CCGATCGCCGGGAGTTCATGTAT

catalase:

For: TTCTGGTTATCCCGTTGAGC

Rev: GGTAATGGCACCAGGAGAAA

hsp60:

For: TGATGCTGATCTCGTCAAGC

Rev: TACTCGGAGGTGGTGTCCTC

ClpX:

For: AAAATGCTCGAAGGCACAGT

Rev: TTGAGACGACGTGCGATAAG

Pink1:

For: TCGGTGGTCAATGTAGTGC

Rev: CCACTCGGAAGATTCCACTGC

BiP:

For: GCTATTGCCTACGGTCTGGA

Rev: CATCACACGCTGATCGAAGT

actin:

For:TTGTCTGGGCAAGAGGATCAG

Rev: ACCACTCGCACTTGCACTTTC

Analysis was performed using the Standard Curve method. Total cDNA concentration was normalized to actin expression. Data are represented as mean ± SEM. 5-6 biological replicates (containing 15-20 heads each) per experiment.

Gaboxadol and Antioxidant Feeding

Gaboxadol hydrochloride (Sigma-Aldrich) was dissolved in water and added to melted cornmeal food to a final concentration of 0.15 mg/mL. Flies were flipped onto Gaboxadol-containing food for 3 days prior to paraquat injection and remained on Gaboxadol-containing food post-injection. Control food was made by adding the

appropriate amount of vehicle alone to melted cornmeal food.

Author Contributions

VMH and MSH conceived of the experiments. Experiments were performed by: VMH (all experiments) and GBS, ISI, and SL (survival of infection, oxidative stress, sleep quantification). VMH, JCC, and MSH made intellectual contributions, designed the figures, and wrote the manuscript.

Acknowledgements

We thank Shirasu-Hiza and Canman lab members for support, productive discussions, and feedback on this manuscript; Reed O'Connor and Charlotte Wayne for technical support; Rodney Rothstein for equipment use; and Nicholas Stavropoulos, Amita Sehgal, Rob Jackson, Paul Shaw, and Jeff Donlea for fly lines. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. This work was supported by: the Amgen Scholars Program (GBS), the I.I. Rabi Scholars Program (ISI), NIH R01GM117407 (JCC); NIH DP2OD008773 (JCC); Hirschl Foundation (MSH); NIH R01GM105775 (MSH); and NIH R01AG045842 (MSH).

Chapter III: Further investigations into the role of ROS in sleep

Introduction

In the previous chapter, a reciprocal relationship between sleep and oxidative stress was demonstrated. Here, I will provide additional data in support of this relationship. I will also address other experiments that were performed to strengthen the key findings in Chapter II, but which posed substantial challenges and will require further exploration. Lastly, I will introduce new data that may add more detail to our understanding of the relationship between sleep and oxidative stress.

Additional support for a relationship between sleep and ROS

Though Chapter II focused on the shared phenotype of oxidative stress sensitivity among short-sleeping flies, these flies were also tested for defects in other physiologies. A global immunity defect was not evident across short-sleeping flies, but a specific alteration in immune function that may have a connection to ROS is discussed in Appendix II (Fig. 3.1). Another physiology that was investigated in short-sleeping flies was heat sensitivity. It has been shown previously that sleep deprivation in rodents results in increased expression of chaperones and heat shock proteins such as *hsp27*, *hsp60*, and *hsp70* [80,167,168]. Additionally, the *Drosophila* heat shock mutant *Hsp83* exhibits an exaggerated sleep rebound following sleep deprivation and dies much earlier than controls from sleep deprivation [64], suggesting a role for heat shock proteins in sleep homeostasis. Thus, I tested short-sleeping flies for sensitivity to hyperthermia; results varied depending on the temperature flies were exposed to, but a

dramatic sensitivity was not observed in all short-sleeping flies. This result confirms that short-sleeping flies do not have a broad sensitivity to different forms of stress.

One caveat to working with short-sleeping *Drosophila* mutants is the strong selective pressure against their mutations; stocks homozygous for mutations that affect sleep amount will often acquire compensatory mutations that mask the sleep phenotype. Thus, it is commonplace for sleep mutant stocks to "lose" their sleep phenotype, and this can happen in as little as a few generations. I experienced this issue firsthand with two sleep mutants, *redeye* and *fumin*. Though these events were frustrating, I observed that oxidative stress sensitivity was lost simultaneously with the loss of the sleep phenotype, further supporting the idea that sleep loss causes sensitivity to oxidative stress. Additional data from the sleep mutant *sleepless*^{P2}, a P-element insertion mutant that I found to have a slight increase in sleep, supports the reciprocal conclusion: that increased sleep promotes resistance to oxidative stress.

In Chapter II, qPCR data from the heads of neuronal *inc-RNAi* flies showed upregulation of antioxidant and mitochondrial stress genes at baseline, suggesting that short-sleeping flies have high levels of ROS in the brain. I also performed qPCR on heads following paraquat injection and found that expression of several of these genes differed in neuronal *inc-RNAi* flies compared to controls, possibly providing an explanation for their early death after oxidative challenge. Moreover, it was demonstrated in Chapter II that reducing ROS levels in the brain by overexpressing neuronal antioxidants results in a reduction in sleep. To further demonstrate the role of ROS in sleep regulation, I reduced ROS levels using an alternative approach—feeding antioxidants—and again, observed a reduction in total sleep.

Challenges Encountered

An obvious way to investigate the relationship between sleep and ROS is to measure ROS levels directly in the brain of a short-sleeping animal. However, direct measurements of ROS have been difficult due to the instability of ROS. ROS-sensitive fluorescent dyes including 2',7'-dichlorofluorescein (H_2DCF) and dihydroethidium (DHE) are used most commonly to measure ROS levels in live tissue either by fluorescent microscopy or by using a fluorimeter to detect levels of fluorescence [169]. A less direct way of investigating the amount of ROS in an organism is to measure oxidative damage caused by ROS in the form of protein carbonyls, lipid peroxides, or oxidized DNA. I took three approaches to measuring ROS in short-sleeping mutants: 1) exposing fly homogenate to H_2DCF in a fluorescent plate reader assay, 2) injecting DHE into live flies and imaging fluorescence through the fly cuticle, and 3) measuring lipid peroxidation in fly homogenate using a TBARS assay. Some of these assays posed challenges or provided confusing data. Thus, additional experiments need to be performed to determine whether ROS levels are altered in the brains of short-sleeping flies.

Chapter II demonstrated that increasing sleep in wild type flies promotes resistance to oxidative challenge. An additional experiment that would strengthen this argument is to rescue sleep by either genetic or pharmacological means in short-sleeping mutants and show a corresponding rescue in survival to oxidative challenge. I attempted the pharmacological approach to this experiment by feeding Gaboxadol to short-sleeping mutants. Though I did find that Gaboxadol increased sleep in all short-

sleeping mutants, I was unable to demonstrate a rescue of survival after paraquat injection. Because lack of movement could result from drug toxicity as well as from increased sleep, Gaboxadol dosage may have been too high in these experiments. Rescuing sleep genetically in sleep mutants may be a better approach.

While sleep amount is manipulated in Chapter II via various genetic and pharmacological methods, a more standard method of sleep manipulation is to mechanically sleep deprive flies [20,64,121]. Because it is difficult to control for the stress induced by mechanical stimulation, which could be independent of the stress induced by sleep reduction, I mechanically sleep deprived control flies alongside neuronal *inc-RNAi* flies and compared their survival after paraquat injection. I encountered some obstacles when optimizing the sleep deprivation set up, and was not able to achieve a significant sleep reduction in one of the two groups of control flies. Nonetheless, I did not find that sleep deprivation induced a change in paraquat sensitivity in neuronal *inc-RNAi* flies or controls.

New Data

Chapter II showed that reducing neuronal ROS levels results in decreased sleep. If ROS levels do indeed reflect sleep need, it is important to also show that increased ROS levels result in prolonged sleep. Increasing ROS levels could be achieved in a tissue specific manner using RNAi against antioxidants, or body-wide by using mutants with defects in oxidative stress response genes. The latter was performed using several mutants with defects in oxidative stress response genes, and a consistent, but somewhat confusing result was observed.

Lastly, it is unclear what causes short-sleeping mutants to succumb early to oxidative challenge. Early death could be due to the effects of oxidative stress on the brain, on the body, or both. If short-sleeping mutants have elevated levels of ROS in the brain, which directly cause sensitivity to oxidative stress, then one might expect reducing ROS levels in the brain via antioxidant overexpression to prolong survival after oxidative challenge. I performed this experiment and observed that neuronal overexpression of *SOD1* or *SOD2* actually resulted in decreased survival to H_2O_2 feeding. This could be due to the reduced sleep caused by neuronal overexpression of antioxidants.

Results

Heat stress sensitivity is not ubiquitous in short-sleeping flies.

I first tested sensitivity to hyperthermia in neuronal *inc-RNAi* flies and found that they have a very subtle sensitivity at 37°C (Fig. 3.2A). This difference was significant compared to both the *elav* control and the *inc-RNAi* control in only 2/4 trials. Because flies exhibit heat paralysis at high temperatures, it was difficult to distinguish dead flies from paralyzed flies. Thus, I repeated these experiments at 33°C and found that neuronal *inc-RNAi* flies were not sensitive to heat stress (Fig. 3.2B). In contrast, neuronal *Cul3-RNAi* flies died dramatically faster than controls at 33° C (Fig. 3.2C). The short-sleeping mutant *redeye* showed a less dramatic, but significant, sensitivity to heat stress at 33° C (Fig. 3.2D). I also performed heat stress assays with *fumin* mutants, which showed no difference in survival compared to controls (data not shown), but it

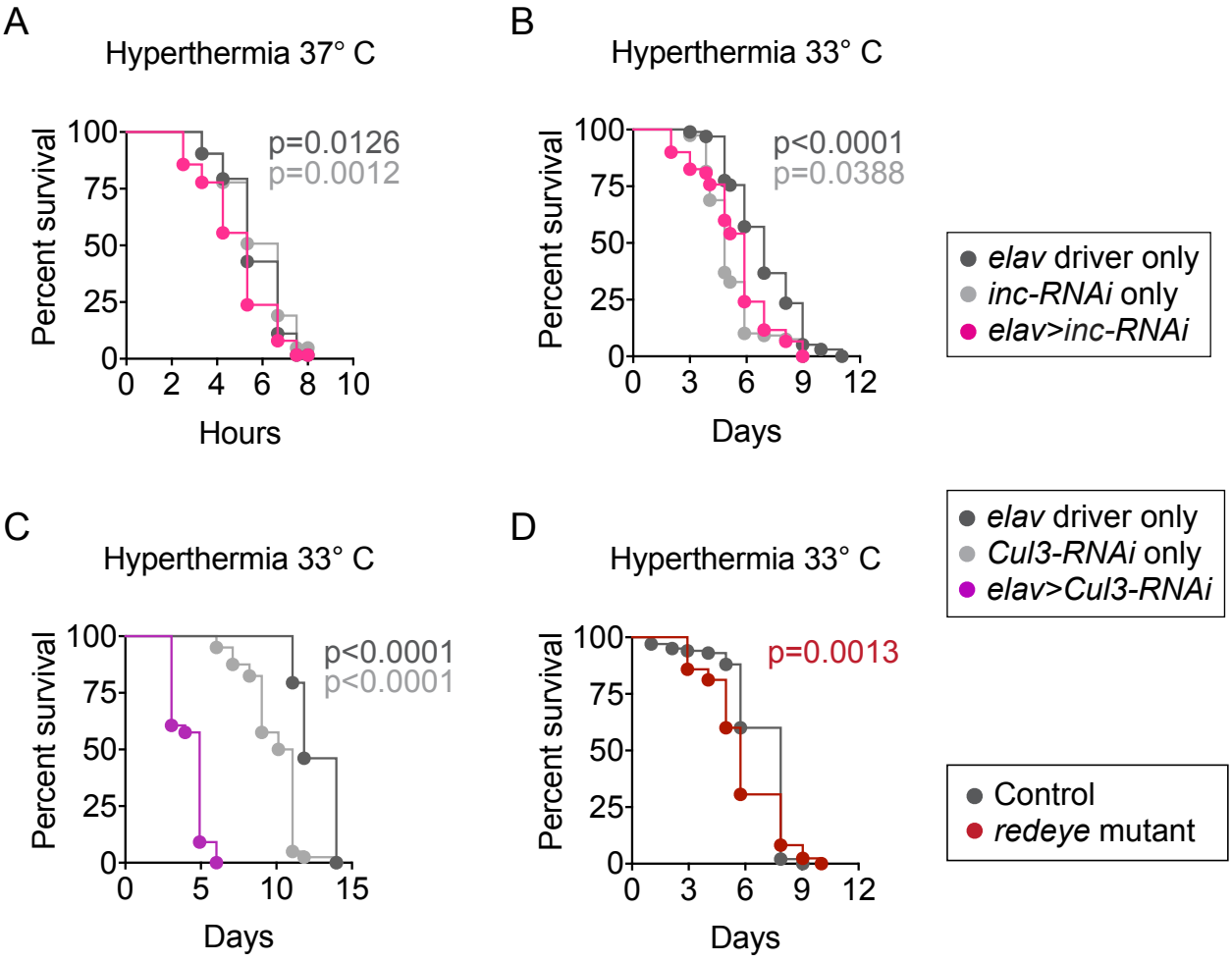

Fig. 3.2. Heat stress sensitivity is not ubiquitous in short-sleeping flies.
Neuronal *inc-RNAi* flies die slightly faster than controls at 37°C (A, $p<0.05$ compared to both controls, n=63 flies/genotype), but die at an intermediate rate relative to controls at 33°C (B, $p<0.0001$ compared to *elav* control, $p=0.0388$ compared to *inc-RNAi* control, n=98-120 flies/genotype). Neuronal *Cul3-RNAi* flies die dramatically faster than both controls at 33°C (C, $p<0.0001$ compared to either control, n=33-40 flies/genotype), while *redeye* mutants demonstrate a more subtle sensitivity to heat relative to controls at 33°C (D, $p=0.0013$, n=85-100 flies/genotype). p-values were obtained by log-rank analysis. Data from representative experiments are shown. All experiments were performed at least twice, with the exception of (D), which was performed only once.

was later confirmed that the *fumin* mutants used in these experiments had lost their sleep phenotype; therefore, these experiments need to be repeated. Overall, heat stress sensitivity is present in some, but not all, short-sleeping flies, indicating that it is not a direct result of sleep reduction.

Loss of sleep phenotype accompanies simultaneous loss of oxidative stress sensitivity.

As demonstrated in Chapter II, *redeye* and *fumin* mutants both show dramatic reductions in sleep relative to controls (Fig. 2.5C-D, left panels) and are both sensitive to oxidative stress by either paraquat injection or H_2O_2 feeding (Fig. 2.5C-D, middle and right panels). At the onset of this project, I was not aware that extensive expansion of homozygous sleep mutant lines, which provides ample opportunity for mutations to arise, can encourage lines to acquire compensatory mutations that mask their sleep phenotype. Some researchers avoid this problem by maintaining the sleep mutations over a balancer chromosome, which prevents crossing over, rather than maintaining homozygous stocks. When I expanded both the *fumin* and *redeye* mutant lines to produce large numbers of flies for experiments, I observed the complete loss (or masking) of the sleep phenotype in both expanded mutant lines compared to flies from their original stocks (Fig. 3.3A-B, left panels).

These expanded *fumin* mutants that lacked a sleep phenotype were no longer sensitive to paraquat injection (Fig. 3.3A, middle panel) or to H_2O_2 feeding (Fig. 3.3A, right panel). H_2O_2 feeding assays were not performed with expanded *redeye* mutants, but it was confirmed via paraquat injection that the *redeye* mutants lacking their sleep

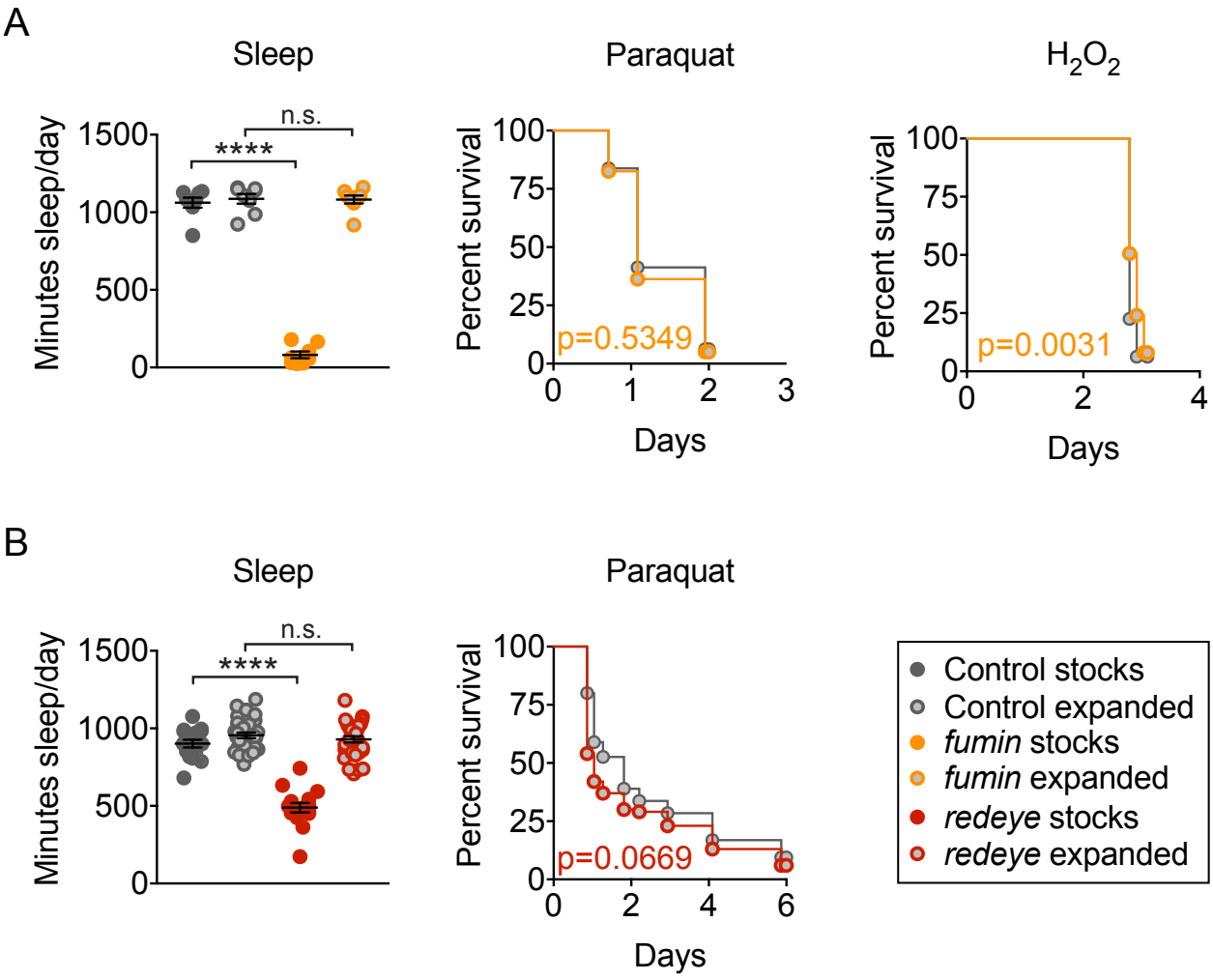

Fig. 3.3. Loss of the sleep phenotype in *fumin* and *redeye* mutants results in simultaneous loss of sensitivity to oxidative stress.
While the original *fumin* (A) and *redeye* (B) mutant stocks still maintain their dramatic sleep reductions compared to control stocks (left panels, solid circles, $p<0.0001$ for both mutant stocks relative to respective control stocks, n=8-16 flies/genotype), expansion of these lines resulted in both *fumin* (A) and *redeye* (B) mutants losing their sleep phenotypes (left panels, outlined circles, $p>0.05$ for both expanded mutants relative to respective expanded controls, n=8-32 flies/genotype). Expanded *fumin* mutants died at the same rate as expanded controls after injection with paraquat (A, middle panel, $p>0.05$) and died slightly slower than expanded controls during H_2O_2 feeding (A, right panel, $p=0.0031$). Expanded *redeye* mutants also died at the same rate as expanded controls after injection with paraquat (B, right panel, $p>0.05$). For scatter plots (left panels), each data point represents the average sleep in minutes/day measured across 5 days for an individual animal. Data are shown as mean ± SEM and p-values were obtained by unpaired students t-test (left panels) or by log-rank analysis (middle and right panels).

phenotype also lost their sensitivity to oxidative stress (Fig. 3.3B, right panel). While the loss of the sleep phenotypes in these mutants was frustrating, and has since been avoided by maintaining these stocks over balancers, these events provided additional evidence that sleep reduction causes sensitivity to oxidative stress.

sleepless^{P2} flies are long-sleeping and resistant to oxidative stress.

In Chapter II, the short-sleeping phenotype of *sleepless$^{\Delta 40}$* mutants was verified (Fig. 2.5B, left panel). *sleepless$^{\Delta 40}$* mutants were the result of an imprecise excision of a P-element inserted into the *sleepless* gene, and mutants containing the original P-element, *sleepless^{P1}*, also sleep less than control flies [36]. Another P-element insertion into the 3'UTR of the *sleepless* locus resulted in *sleepless^{P2}* flies, which were previously shown to have wildtype or very slightly reduced levels of sleep [36]. However, when I measured sleep in *sleepless^{P2}* mutants, I found that they slept more than control flies (Fig. 3.4A). Additionally, *sleepless^{P2}* mutants died slower than controls after paraquat injection (Fig. 3.4B), further supporting the conclusion from Chapter II that increased sleep promotes resistance to oxidative challenge.

Neuronal *inc-RNAi* flies have altered gene expression following paraquat injection.

To determine whether early death after oxidative challenge in neuronal *inc-RNAi* flies is caused by altered expression of cytoprotective genes in the brain, I performed qPCR on the heads of neuronal *inc-RNAi* and control flies following paraquat injection. I measured expression of the same antioxidant, mitochondrial stress response, and ER

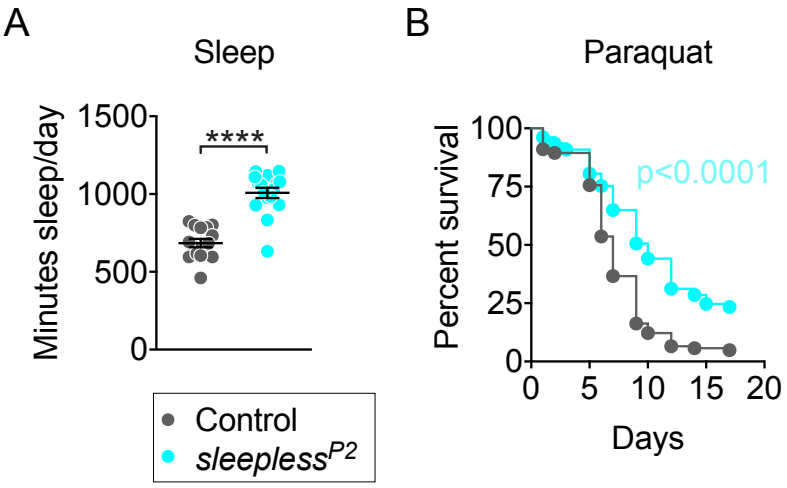

Fig. 3.4. *sleepless*^{P2} mutants have prolonged sleep and are resistant to oxidative stress.
sleepless^{P2} mutants sleep about 30% more than control flies (A, p<0.0001, n=15-16 flies/genotype) and die slower than controls after injection with paraquat (B, p<0.0001, n=77-123 flies/genotype). For scatter plot (A), each data point represents the average sleep in minutes/day measured across 5 days for an individual animal. Data are shown as mean ± SEM. p-values were obtained by unpaired students t-test (A) or by log-rank analysis (B). Data from representative experiments are shown. Experiments were performed at least twice.

stress response genes measured at baseline in Chapter II (Fig. 2.7A). For many of these genes, there was no difference in paraquat-induced gene expression between neuronal *inc-RNAi* flies and controls (Fig. 3.5 A-D). In contrast, while controls showed increased expression of *catalase* and *hsp60* after paraquat injection, neuronal *inc-RNAi* flies failed to respond to paraquat with any change in gene expression (Fig. 3.5 E-F). Moreover, while control flies showed no change in *ClpX* and *Pink1* expression after paraquat injection, neuronal *inc-RNAi* flies exhibited decreased expression after paraquat injection (Fig. 3.5 G-H). This failure to properly express protective stress response genes in the head following paraquat injection may explain why neuronal *inc-RNAi* flies succumb earlier than controls after acute oxidative stress.

Antioxidant feeding reduces sleep amount.

In Chapter II, overexpressing antioxidants in neurons caused a reduction in sleep (Fig. 2.8). To verify this result using a pharmacological approach, I fed wildtype flies one of two antioxidants: N-acetylcysteine, which replenishes glutathione levels in the body, or melatonin, a potent endogenous antioxidant. Melatonin feeding resulted in an 8% decrease in sleep (Fig. 3.6) and NAC feeding caused a similar, but not significant, trend (Fig. 3.6). These results suggest that decreasing ROS levels by dietary antioxidants causes a small reduction in total sleep amount.

Measuring ROS levels in short-sleeping flies.

My first approach to measuring ROS in short-sleeping flies was to use the ROS-sensitive dye H_2DCF, which reacts with H_2O_2 to form a fluorescent green product. While

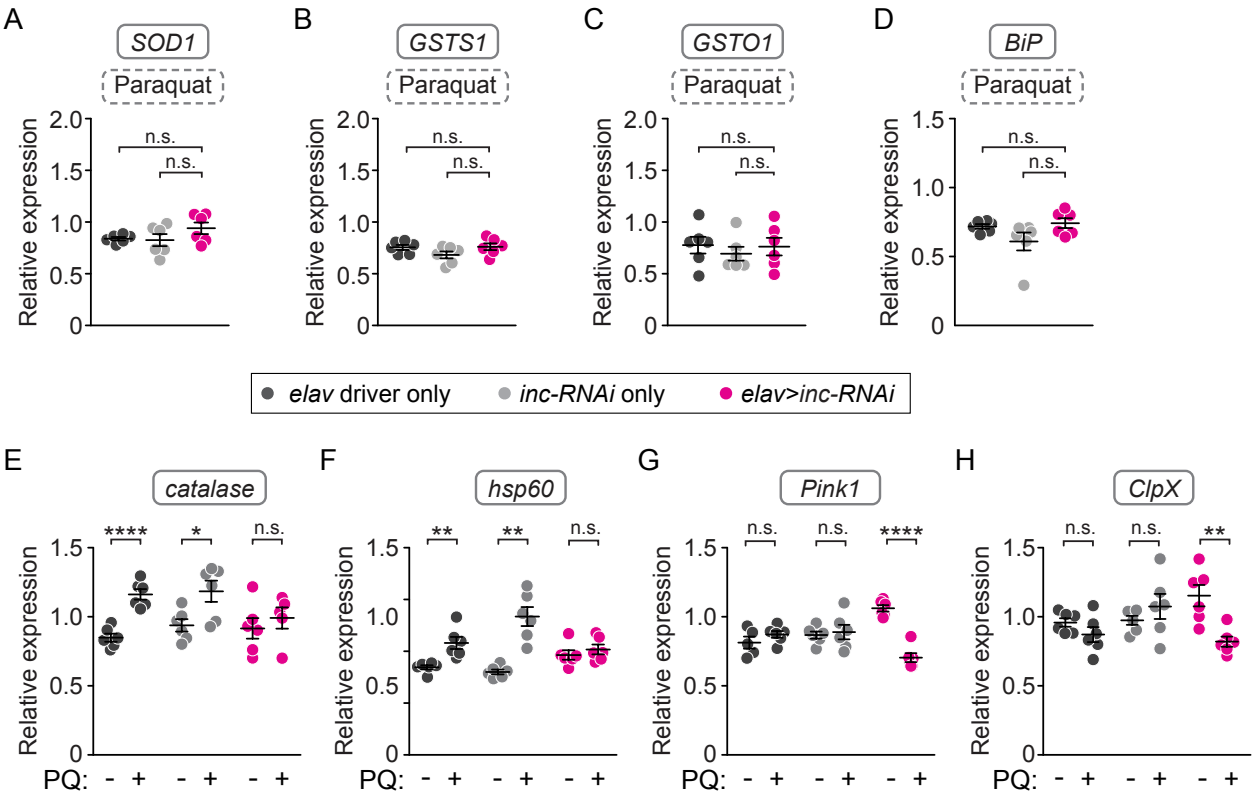

Fig. 3.5. Neuronal *inc-RNAi* flies show altered expression of stress response genes following paraquat injection. After paraquat injection, neuronal *inc-RNAi* flies and controls express similar levels of the following genes in the head: *SOD1* (A), *GSTS1* (B), GSTO1 (C), and *BiP* (D) ($p>0.05$ for each gene relative to either control, n=6 biological replicates/genotype). While controls respond to paraquat injection with increased head expression of both *catalase* (E, $p<0.05$ relative to either control, n=6 biological replicates/genotype) and *hsp60* (F, $p<0.01$ relative to either control, n=6 biological replicates/genotype) relative to baseline expression, neuronal *inc-RNAi* flies do not show a significant change in expression of either *catalase* (E, $p>0.05$, n=5 biological replicates/conditoin) or *hsp60* (F, $p>0.05$, n=6 biological replicates/condition). Following paraquat injection, control expression of the mitochondrial stress genes *Pink1* (G, $p>0.05$ relative to either control, n=5-6 biological replicates/condition) and ClpX (H, $p>0.05$ relative to either control, n=6 biological replicates/condition) is comparable to baseline levels in the head, while neuronal *inc-RNAi* expression of both *Pink1* (G, $p<0.0001$, n=6 biological replicates/condition) and *ClpX* (H, $p<0.01$, n=6 biological replicates/condition) is reduced compared to baseline levels in the head. Expression is normalized to *actin*. Data are shown as mean ± SEM. Each data point represents an independent biological replicate with 15-20 individual fly heads per biological replicate. p-values were obtained by ordinary one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons.

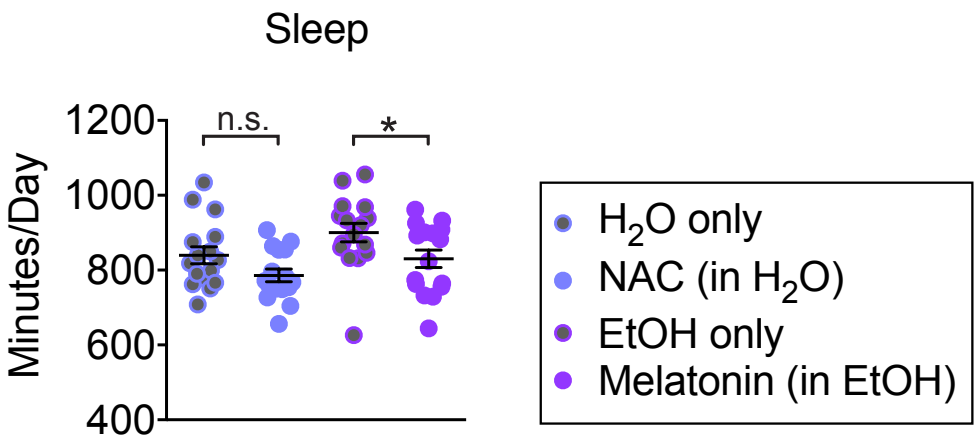

Fig. 3.6. Antioxidant feeding in wildtype flies reduces sleep.
Wildtype flies were fed food mixed with NAC (dissolved in water), or food mixed with water alone. NAC fed flies slept about 6% less than control flies, though the difference did not reach significance (p=0.0677, n=16 flies/condition). Alternatively, wildtype flies were fed food mixed with melatonin (dissolved in ethanol) or food mixed with ethanol alone. Melatonin fed flies slept about 8% less than controls (p=0.0499, n=16 flies/condition). Each data point represents the average sleep in minutes/day measured across 5 days for an individual animal. Data are shown as mean ± SEM. p-values were obtained by unpaired students t-test. Data from a representative experiment are shown. Experiment was performed twice.

H_2DCF is commonly thought of as a specific indicator of H_2O_2, it can be oxidized by other forms of ROS as well, so it is more accurate to consider it a general marker for oxidative stress [169]. Because the majority of ROS in the body is produced by mitochondria [170], I separated fly homogenate into mitochondrial and cytosolic fractions. I added H_2DCF dye and respiration buffer, containing mitochondrial substrates to promote respiration, to the mitochondrial and cytosolic fractions from neuronal *inc-RNAi* and control heads and then measured fluorescence using a fluorescent plate reader. I found that neuronal *inc-RNAi* heads had slightly higher ROS levels in the cytosolic fraction relative to controls (Fig. 3.7A, left panel), consistent with the idea that short-sleeping flies have high baseline levels of ROS in the brain. This trend was contrasted by slightly lower levels of ROS in the mitochondrial fraction of neuronal *inc-RNAi* heads, though this difference was not significant compared to the *elav* control (Fig. 3.7A, right panel). Lower ROS in the mitochondrial fraction was unexpected, but may indicate that neuronal *inc-RNAi* flies have damaged mitochondria that are unable to respire normally.

To determine whether neuronal *inc-RNAi* flies have higher levels of oxidative damage in the brain caused by chronically elevated ROS, I measured lipid peroxidation. ROS react with poly unsaturated fatty acids to form unstable peroxides that break down into a number of compounds, including malondialdehyde (MDA). MDA reacts with thiobarbituric acid to form an MDA-adduct, which can be quantified colorimetrically [171]. I measured baseline MDA levels in whole body (3.7B, left panel) and heads (3.7B, right panel) and found in both cases that neuronal *inc-RNAi* flies had MDA levels comparable to controls.

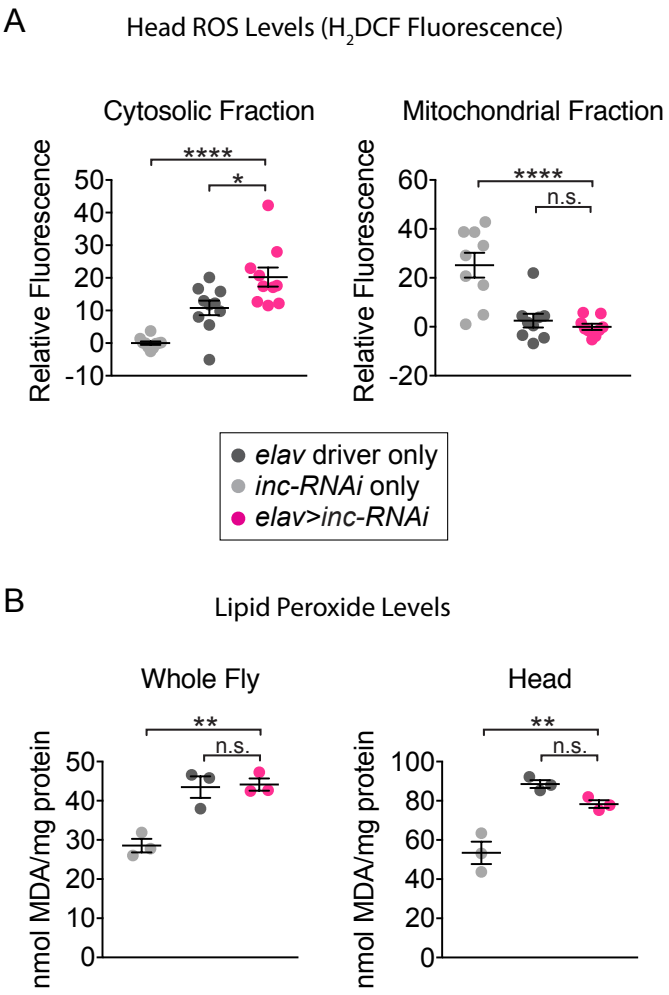

Fig. 3.7 Measuring ROS and oxidative damage in neuronal *inc-RNAi* flies. ROS-sensitive H_2DCF dye was added to fly homogenate from neuronal *inc-RNAi* and control heads that had previously been separated into cytosolic (A, left panel) and mitochondrial (A, right panel) fractions. Compared to controls, neuronal *inc-RNAi* flies had elevated fluorescence, indicating higher ROS, in the cytosolic fraction ($p<0.05$ relative to either control, n=10 biological replicates/genotype), and fluorescence in the mitochondrial fraction that was comparable to the *elav* control but lower than the *inc-RNAi* control ($p>0.05$ relative to *elav* control, $p<0.0001$ relative to *inc-RNAi* control, n=9 biological replicates/genotype). Pooled data from 3 independent experiments are shown; to correct for variation in fluorescence range between experiments, data was normalized to the genotype with the lowest average value per experiment (A). MDA content was measured as an indicator of lipid peroxide levels in the whole fly (B, left panel), or heads (B, right panel). In both cases, neuronal *inc-RNAi* flies had MDA levels comparable to the *elav* control, and elevated compared to the *inc-RNAi* control ($p>0.05$ relative to *elav* control, $p<0.01$ relative to *incRNAi* control, n=3 biological replicates/genotype). Data from a single experiment are shown (B). All data are shown as mean ± SEM. Each data point represents an independent biological replicate containing 30-40 heads, or 30 whole flies. p-values were obtained by ordinary one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons.

Lastly, I used the ROS-sensitive dye DHE to measure ROS levels in live flies. DHE reacts specifically with superoxide radicals to form a red fluorescent product that intercalates with DNA [169]. Due to its specificity and its ability to stay within cells, DHE is considered a less problematic alternative to H_2DCF. I injected DHE into live neuronal *inc-RNAi* and control flies and imaged through the dorsal cuticle of their abdomen. Variability was high between individual flies, but when data was pooled from multiple experiments I observed a very small increase in neuronal *inc-RNAi* flies (Fig. 3.8A, left panel). A similar, but not significant, trend was observed when neuronal *inc-RNAi* flies were injected with Alexa568 (Fig. 3.8A, right panel), which I used as a control dye because it has a similar emission wavelength to DHE. The subtle difference in fluorescence after injection with the control dye indicates that any differences observed after injection with DHE may be artificial – possibly resulting from smaller body volume in neuronal *inc-RNAi* flies.

I repeated these experiments in short-sleeping *redeye* mutants and observed no difference between mutants and controls when injected with either DHE or the control dye (Fig. 3.8B). When I instead performed these experiments with the dramatically short-sleeping mutant *fumin*, I found that *fumin* mutants had significantly higher ROS levels (Fig. 3.8C, left panel), however they also had significantly higher signal when injected with the control dye (Fig. 3.8C, middle panel). Since the same amount of dye is injected into each fly, I would expect smaller flies to have more concentrated dye and thus exhibit higher fluorescent signal. However, *fumin* mutants look slightly larger than controls by eye, and when I measured their mass I found that they were indeed trending larger (Fig. 3.8C, right panel), though the difference was not significant. It is unclear

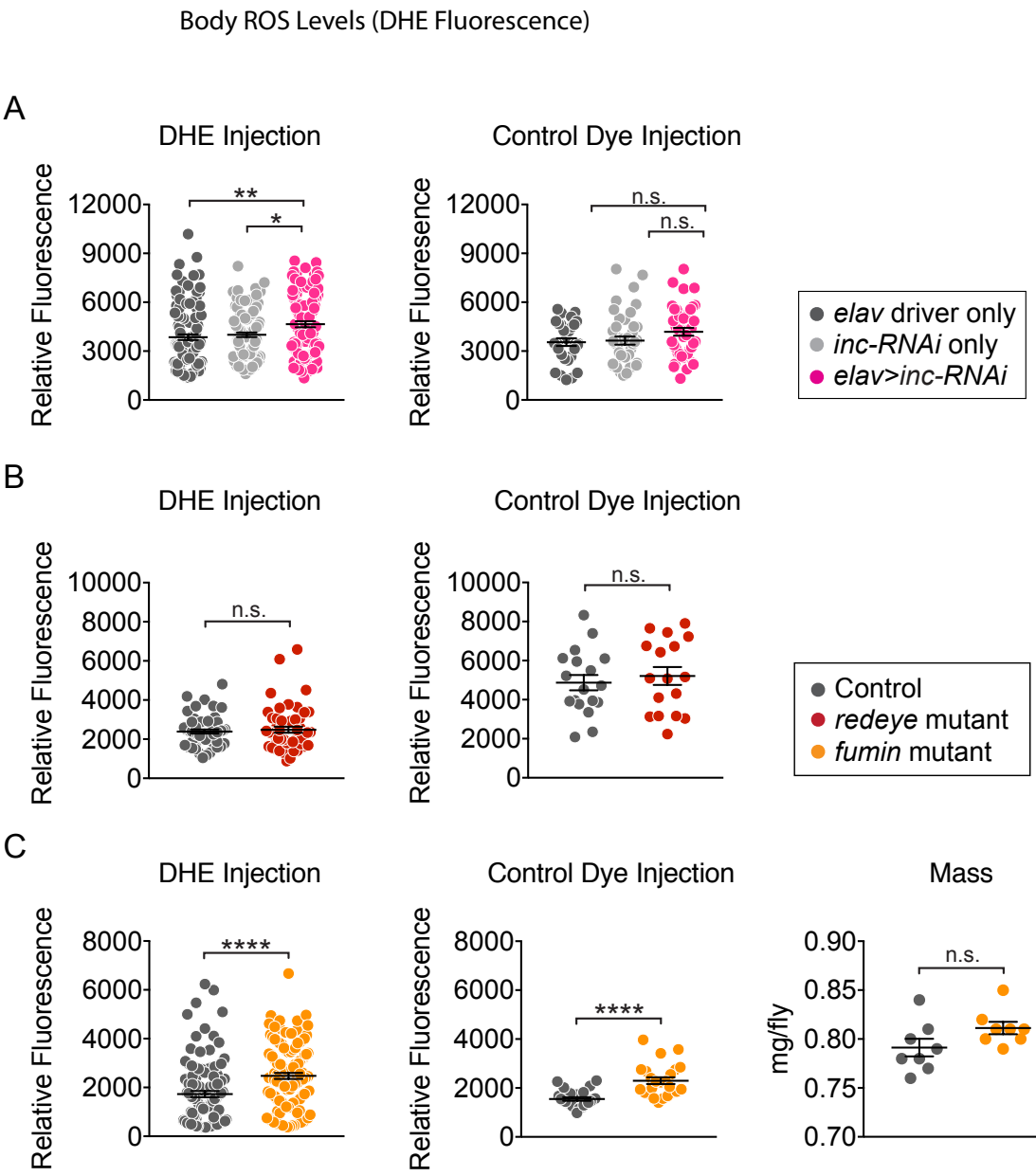

Fig. 3.8. ROS measurements in live short-sleeping flies by DHE injection.
Live flies were injected with DHE and imaged through the dorsal cuticle of the abdomen. After DHE injection, neuronal *inc-RNAi* flies exhibited slightly higher fluorescence relative to controls ($p<0.05$ relative to either control, n=93-120 flies/genotype, pooled data from 5 independent experiments are shown), and showed a similar but not significant trend when injected with the control dye, Alexa568 ($p>0.05$ relative to either control, n=30-42 flies/genotype, pooled data from 2 experiments independent experiments are shown). *Redeye* mutants exhibited no difference in fluorescence after injection with either DHE ($p>0.05$, n=53-55 flies/genotype, pooled data from 2 independent experiments are shown) or Alexa568 ($p>0.05$, n=17-18 flies/genotype, data from a single experiment are shown). *fumin* mutants showed higher fluorescence after either DHE injection ($p<0.0001$, n=108-120 flies/genotype, pooled data from 4

independent experiments are shown) or Alexa568 ($p<0.0001$, n=24 flies/genotype, data from a single experiment are shown). Increased fluorescence even with the control dye cannot be explained by smaller size in *fumin* mutants, as indicated by their mass, which is comparable (though trending larger) to controls ($p=0.09$, n=80 flies/genotype, data from a single experiment are shown. All data are shown as mean ± SEM Each data point represents an individual animal, expect for C (right panel), where each data point represents a group of 10 flies. p-values were obtained by ordinary one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons (A), or by unpaired students t-test (B-C).

what is causing the difference in fluorescence after injection with control dye, but it may not be coincidence that the same problem occurred in both neuronal *inc-RNAi* flies and *fumin* mutants. One possibility is that the control dye is also sensitive to oxidation, like DHE.

Gaboxadol feeding rescues sleep in short-sleeping flies, but may not rescue sensitivity to oxidative stress.

In Chapter II, wild type flies were fed the sleep-inducing drug Gaboxadol, which resulted in extended survival after paraquat injection compared to vehicle-fed controls (Fig. 2.6B). I wondered whether Gaboxadol treatment would rescue the short sleep phenotype of short-sleeping flies, as well as rescue their corresponding oxidative stress sensitivity.

First, I investigated Gaboxadol's effect on sleep in short-sleeping flies. I found that Gaboxadol feeding increased sleep in neuronal *inc-RNAi* flies nearly to the same levels as controls, but there was still a slight difference between neuronal *inc-RNAi* flies and controls during Gaboxadol treatment (Fig. 3.9A). In the case of *sleepless* mutants, Gaboxadol-feeding increased sleep in both mutants and controls, such that sleep in Gaboxadol-fed *sleepless* flies was comparable to Gaboxadol-fed controls (Fig. 3.9B). However in *fumin* mutants, only a partial rescue was observed (Fig. 3.9C). Half of the *fumin* mutants failed to respond to the drug, while the other half showed sleep levels comparable to vehicle fed controls. The subset of *fumin* mutants that responded to the drug still slept less than Gaboxadol-fed controls. Lastly, *redeye* mutants showed a surprisingly large response to Gaboxadol, surpassing the sleep amount of drug-fed

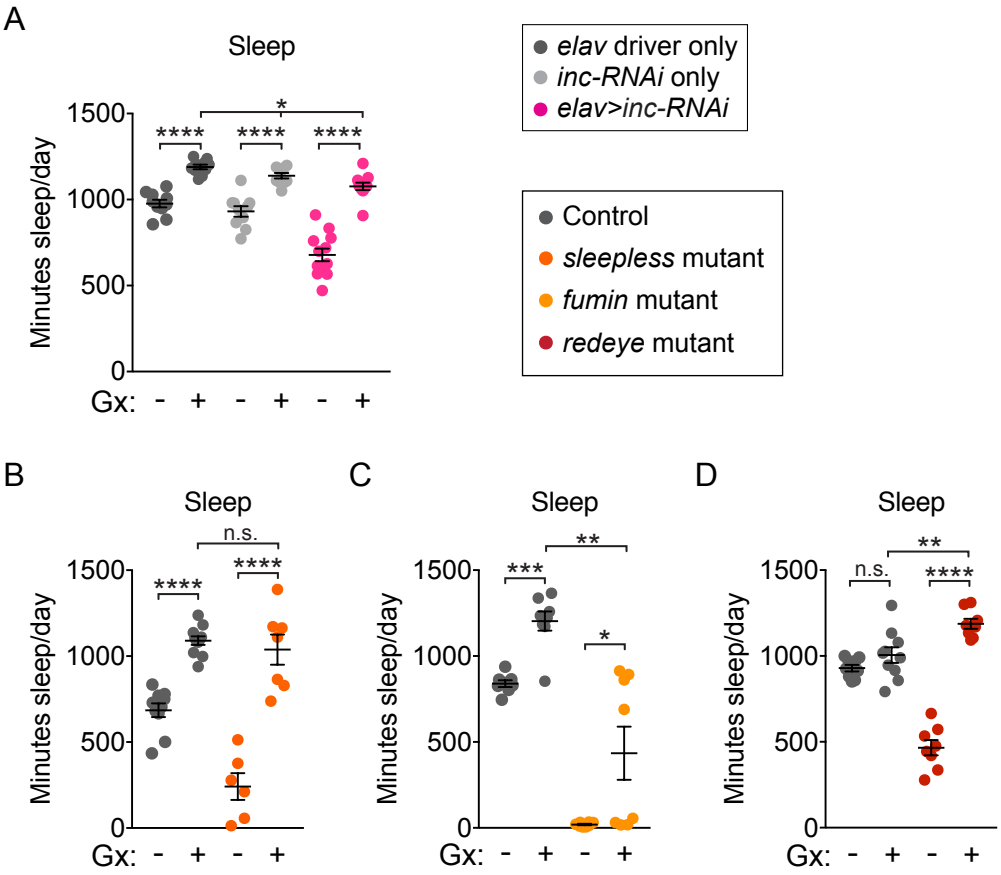

Fig. 3.9. Gaboxadol feeding rescues sleep to varying degrees in several short-sleeping flies.
Gaboxadol (Gx) feeding increased sleep in neuronal *inc-RNAi* flies and controls relative to vehicle-fed counterparts (A, $p<0.0001$ for each genotype, n=10-12 flies/genotype), but there was still a significant difference in sleep between Gx-fed *inc-RNAi* flies and G-fed controls ($p<0.05$ relative to either control). Gx-feeding increased sleep in both *sleepless* mutants and controls relative to vehicle-fed counterparts (B, $p<0.0001$ for each genotype, n=6-11 flies/genotype), and there was no difference in sleep between Gx-fed *sleepless* flies and Gx-fed controls ($p>0.05$). While Gx-feeding resulted in a significant increase in sleep in both *fumin* mutants and controls relative to vehicle-fed counterparts (C, $p<0.05$ for each genotype, n=8 flies/genotype), only a subsut of *fumin* mutants responded to the drug and there was still a significant difference in sleep between Gx-fed *fumin* mutants and Gx-fed controls ($p<0.01$). Lastly, Gx-fed *redeye* mutants showed a robust increase in sleep compared to vehicle-fed counterparts, while Gx-fed controls did not show a significant increase in sleep (D, $p<0.0001$ for *redeye*, $p>0.05$ for control, n=8-10 flies/genotype). This difference in response to Gx resulted in Gx-fed *redeye* mutants sleeping more than Gx-fed controls ($p<0.01$). Each data point represents the average sleep in minutes/day measured across 5 days for an individual animal. Data are shown as mean ± SEM. p-values were obtained by one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons (A), or by unpaired students t-test by unpaired students t-test. Data from a representative experiment are shown. Experiments were performed twice.

controls, which were the only group that did not show a significant increase in sleep from Gaboxadol treatment (Fig. 3.9D).

Next I subjected Gaboxadol-fed *inc-RNAi* flies and controls to paraquat injection, to determine whether sleep rescue could also rescue the oxidative stress sensitivity of *inc-RNAi* flies. I tested the same experimental paradigm previously used with wild type flies in Chapter II (Fig. 2.6B): flies were fed Gaboxadol for 3 days, injected with paraquat, and returned to fresh Gaboxadol food for the remainder of the experiment. With neuronal *inc-RNAi* flies and controls, I found that there was a noticeable collapse in survival curves between the Gaboxadol-fed groups (Fig. 3.10B) compared to the robust difference seen in the vehicle-fed groups (Fig. 3.10A). However, there was not a global shift toward increased survival after paraquat injection in the Gaboxadol-fed flies, as was previously seen in wild-type flies exposed to the same experimental conditions (Fig. 2.6B). While Gaboxadol-fed neuronal *inc-RNAi* flies showed a very slight and not significant survival advantage over their vehicle-fed counterparts, both Gaboxadol-fed controls showed decreased survival relative to their vehicle-fed counterparts. This result is not consistent with the effect of Gaboxadol in wild type flies. Different genetic backgrounds likely vary in their sensitivity to Gaboxadol; it is possible that the dosage of the drug used in these experiments was causing toxicity in the neuronal *inc-RNAi* flies and controls. Toxicity would not be detected by sleep analysis since it likely causes reduced movement, as does increased sleep.

Mechanical sleep deprivation

Mechanical disruption of sleep by repeated shaking or tapping is the standard method

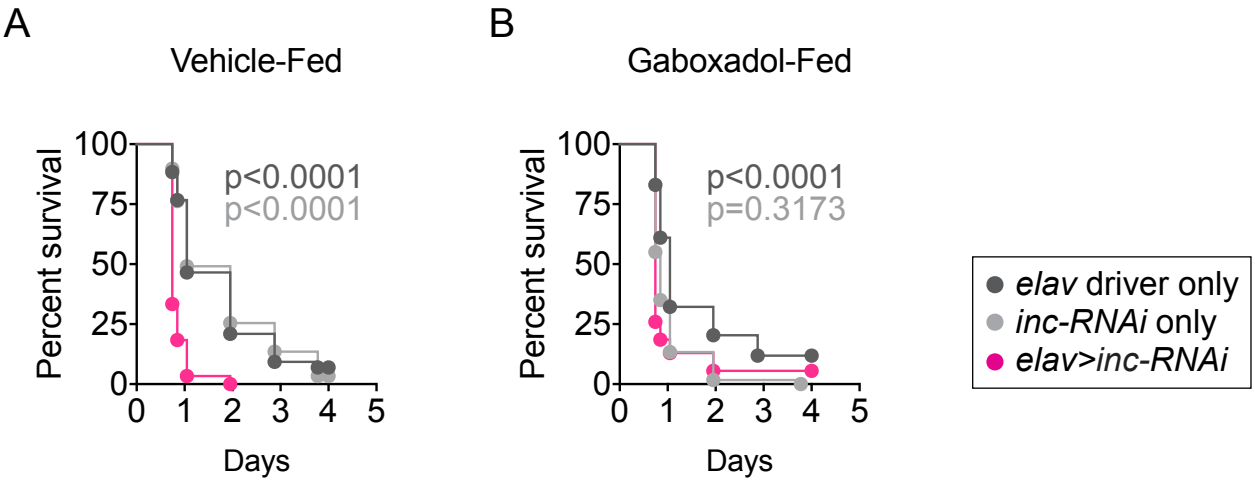

Fig. 3.10. Gaboxadol-feeding collapses difference in survival after PQ injection in neuronal *inc-RNAi* flies.
While vehicle-fed neuronal *inc-RNAi* flies died faster than controls after paraquat injection (A, p<0.0001 relative to either control, n=43-60 flies/genotype), Gaboxaol-fed neuronal *inc-RNAi* flies died at the same rate as *incRNAi* controls and at a faster rate than *elav* controls (B, p<0.0001 relative to *elav* control, p=0.3173 compared to *inc-RNAi* control, n=54-60 flies/genotype). Gaboxadol-feeding does not confer a significant survival advantage after PQ injection in these flies. p-values were obtained by log-rank analysis. Data from representative experiments are shown. Experiments were performed three times.

of sleep deprivation in the fruit fly. Since the short-term feeding of Gaboxadol in wildtype flies was sufficient to promote resistance to oxidative stress (Fig. 2.6B), I wondered whether subjecting wildtype flies to short term sleep deprivation would be sufficient to cause oxidative stress sensitivity. Because this method of sleep deprivation induces stress that could act as a confounding variable, I chose to subject short-sleeping neuronal *inc-RNAi* flies to mechanical sleep deprivation (which should presumably have little effect) and compare their survival after paraquat injection to the survival of mechanically sleep deprived control flies (*elav* and *inc-RNAi* controls). As an additional control, I also performed paraquat injections on unmolested (not mechanically disturbed) neuronal *inc-RNAi* flies and controls to allow comparison of death rates between the two conditions.

First, I optimized the mechanical sleep deprivation system such that control flies slept about as much as neuronal *inc-RNAi* flies. To achieve this, flies were attached to a vortexer that shook repeatedly over the full 12 hours of their subjective night. I found that shaking flies for periods any longer than 12 hours was ineffective because flies began to sleep even during shaking. Using this experimental set up, 12 hours of sleep deprivation resulted in no significant difference in nighttime sleep between sleep-deprived neuronal *inc-RNAi* flies and controls (Fig. 3.11A, black bar); however, the sleep reduction in the *inc-RNAi* controls was minor, and often too subtle to reach significance compared to sleep in unmolested *inc-RNAi* controls (Fig. 3.11A, light gray bar). Thus, for these experiments, *elav* controls serve as a better representation of sleep-deprived flies than the *inc-RNAi* controls.

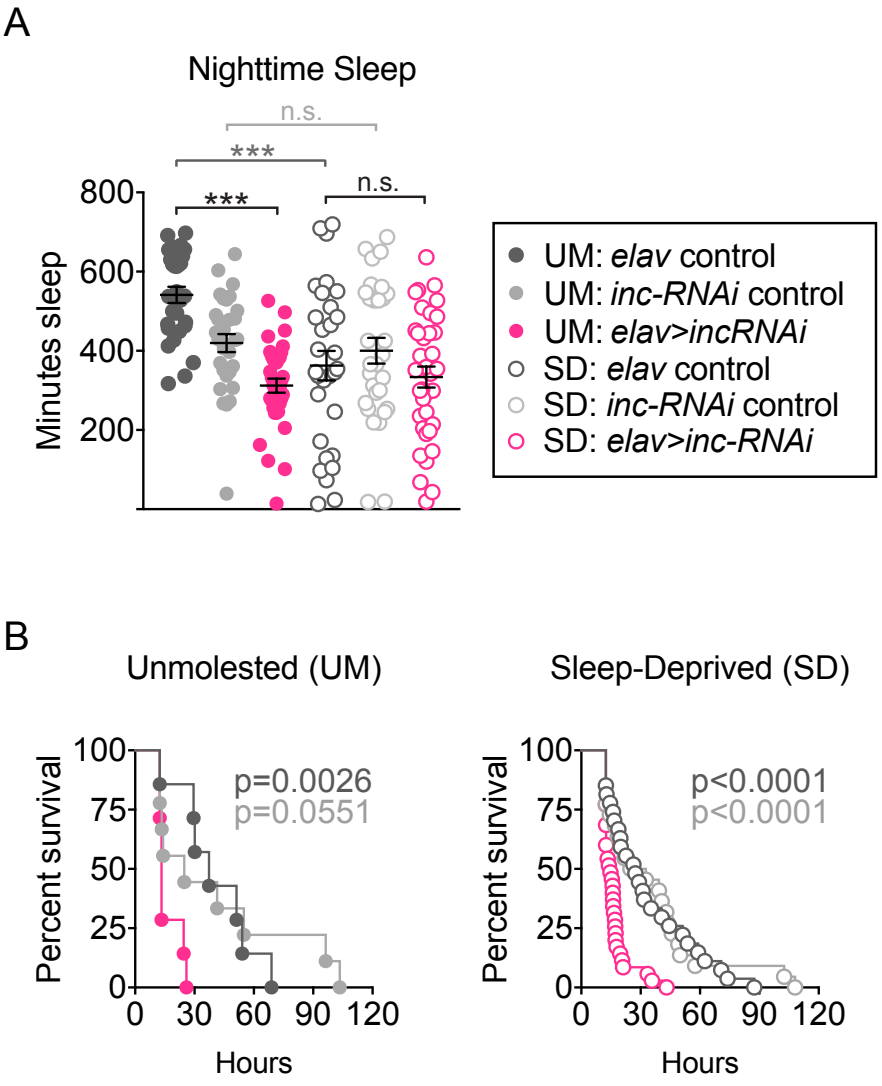

Fig. 3.11. One night of mechanical sleep deprivation does not cause sensitivity to oxidative stress. Unmolested (UM) neuronal *inc-RNAi* flies sleep significantly less than their UM controls during the fly's subjective night (A, p<0.001 relative to either control, n=29-36 flies/genotype). 12 hours of mechanical sleep-deprivation (SD) results in no significant difference in nighttime sleep amount between neuronal *inc-RNAi* flies and controls (A, p>0.05 relative to either control, n=30-36 flies/genotype). While SD *elav* controls sleep significantly less than UM *elav* controls (A, p<0.001, dark gray bar), SD *inc-RNAi* controls do not have a significant reduction in sleep compared to UM *inc-RNAi* controls (A, p>0.05, light gray bar). Paraquat injected UM flies (B, left panel, n=7-9 flies/genotype) die at a similar rate to paraquat-injected SD flies (B, right panel, n=30-34), and in both conditions neuronal *inc-RNAi* flies die faster than controls. For scatter-plot (A), each data point represents the number of minutes slept by an individual fly in a single night; data are shown as mean ± SEM. p-values were obtained by one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons (A), or by log-rank anlaysis (B). Data from a representative experiment are shown. Experiments were performed three times.

To determine whether sleep-deprived control flies become sensitized to oxidative stress, I injected neuronal *inc-RNAi* and control flies with paraquat just before the start of the subjective night; half of the flies were then sleep-deprived while the other half were left unmolested. I found that the sleep-deprived flies had very similar death rates compared to their unmolested counterparts following sleep deprivation (Fig. 3.11B). This result suggests that 12 hours of mechanical sleep deprivation is not sufficient to induce oxidative stress sensitivity.

Reduced sleep in stress response mutants.

Since reducing ROS levels in the brain caused a reduction in sleep (Fig. 2.8B), I wondered if mutants with altered ROS levels would also have altered sleep. I measured sleep in flies carrying mutations in genes that are induced by ROS: *nrf2,* an important antioxidant transcription factor, *GSTS1*, a major antioxidant, and *hsp60*, a mitochondrial UPR chaperone. I found that each of these mutants had significantly reduced total sleep relative to controls (Fig. 3.12A). I also found that these mutants slept in short bouts, displaying a reduced average bout length compared to controls (Fig. 3.12B), which accounted for their reduction in total sleep. Though they slept less, these mutants initiated more sleep bouts per day than control flies (Fig. 3.12C), indicating a defect in sleep maintenance. I observed a similar pattern in a number of other stress response mutants (Appendix III, Table 3.1).

While these results are striking, I found that it was much less straightforward to determine in what way ROS levels were altered in these mutants. I had originally expected each mutant to be sensitive to oxidative stress, indicative of higher ROS

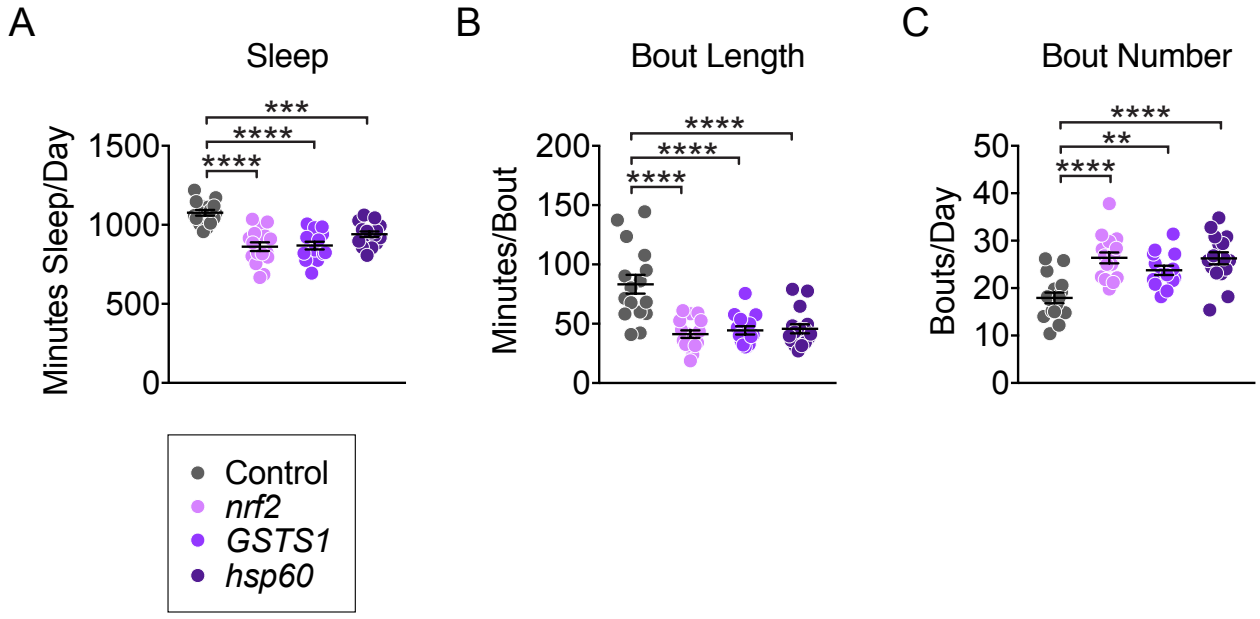

Fig. 3.12. Altered sleep in stress response mutants. Flies with mutations in three genes that respond to high ROS levels—*nrf2*, *GSTS1, hsp60*—show the following alterations in sleep: reduced total daily sleep (A, $p<0.001$), reduced average sleep bout length (B, $p<0.0001$), and increased total daily sleep bout number (C, $p<0.01$). Each data point represents the measurement in a single fly averaged across 5 days. Data are shown as mean ± SEM. p-values were obtained by unpaired students t-test by unpaired students t-test. Data from a representative experiment are shown. Experiments were performed at least three times.

levels, because they each have defects in responses that are induced by ROS. However, oxidative stress assays yielded variable results (Appendix III, Table 3.2) possibly due to compensatory induction of oxidative stress responses that resulted in a survival advantage in response to moderate oxidative challenge, but a disadvantage in response to stronger oxidative challenge.

Neuronal antioxidant overexpression causes sensitivity to oxidative stress.

In order to determine whether reduced ROS in the brain has an effect on survival after oxidative challenge, I performed a H_2O_2 feeding assay on flies overexpressing neuronal *SOD1, SOD2,* or *catalase* (*cat*). Interestingly, neuronal overexpression of *SOD1* and *SOD2* both caused sensitivity to H_2O_2 feeding (Fig. 3.13 A-B), while neuronal *cat* overexpression had little effect (Fig. 3.13 C). This result suggests that ROS levels in the brain are not important for the body's survival to oxidative challenge, but rather, that sleep reduction caused by reduced ROS in the brain may influence susceptibility to oxidative challenge in the body.

Discussion

This chapter provides additional support to some of the main conclusions made in Chapter II. Here, I showed that short-sleeping mutants are not sensitive to heat stress (Fig. 3.2), suggesting that sleep plays a specific role in oxidative stress, rather than a broad role in various stress responses. Moreover, simultaneous loss of the sleep phenotype and oxidative stress sensitivity in *fumin* and *redeye* mutants (Fig. 3.3) emphasizes that short sleep causes sensitivity to oxidative stress. Contrarily, long-

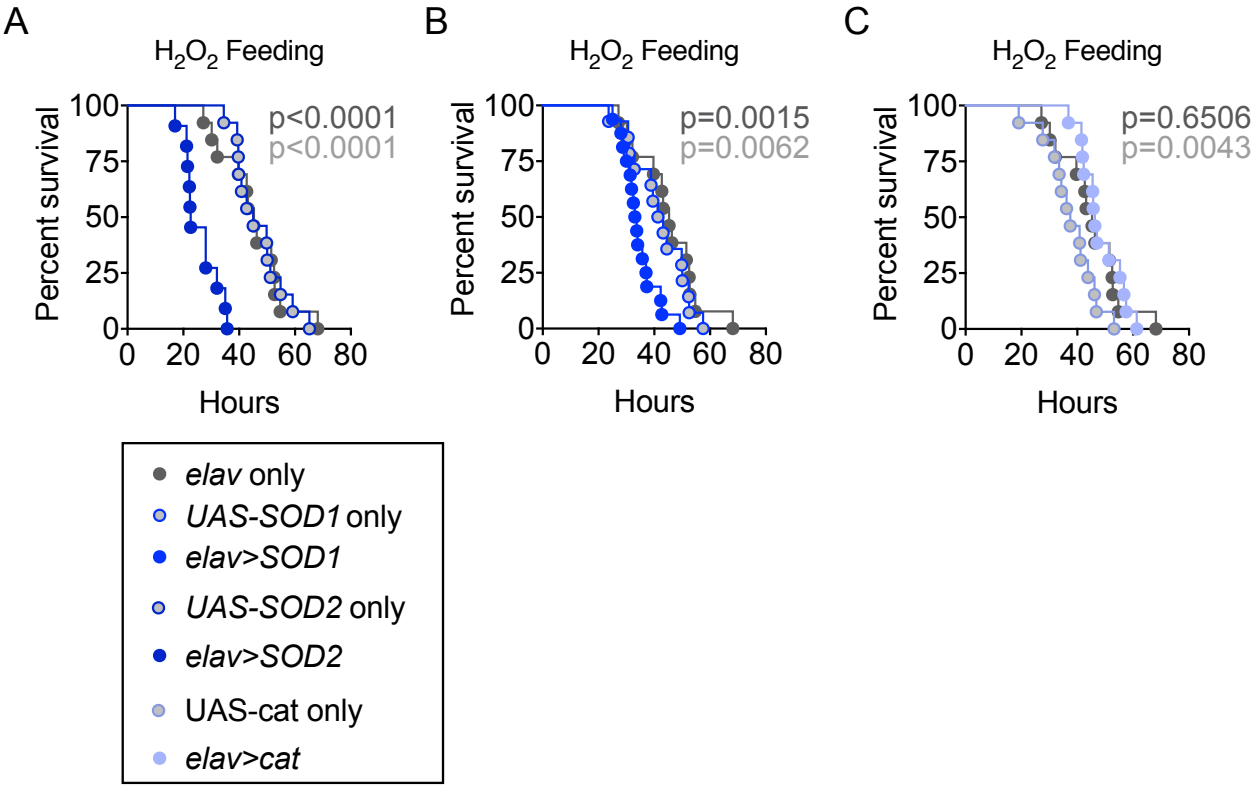

Fig. 3.13 Neuronal overexpression of *SOD1* and *SOD2* causes sensitivity to oxidative stress.
The pan-neuronal driver *elav* was used to overexpress antioxidants *SOD1, SOD2,* and *cat* in the brain. Neuronal overexpression of *SOD1* (A) and *SOD2* (B) resulted in faster death from H_2O_2 feeding ($p<0.01$ relative to either control). Neuronal overexpression of *cat* resulted in no difference in death rate compared to the *elav* control, and faster death compared to the *UAS-cat* control (C, $p<0.01$ relative to *elav* control, $p>0.05$ relative to *UAS-cat* control). p-values were obtained log-rank analysis. Data from a single experiment are shown.

sleeping *sss*^{P2} mutants add to the evidence that increased sleep promotes survival to oxidative challenge (Fig. 3.4). Altered expression of antioxidant and mitochondrial stress genes in the heads of neuronal *inc-RNAi* flies following paraquat injections provides a potential explanation for sensitivity to oxidative challenge (Fig. 3.5). Lastly, antioxidant feeding reduces sleep amount (Fig. 3.6), supporting the conclusion that ROS plays a role in sleep regulation.

This chapter also discussed experimental challenges and data that were more difficult to interpret. Direct measurements of ROS by H_2DCF indicate slightly elevated ROS levels in the brain of neuronal *inc-RNAi* flies (Fig. 3.7), but this result is specific to the cytosolic fraction, and DHE injection experiments failed to clarify whether ROS levels are altered in the body of short-sleeping flies (Fig. 3.8). Gaboxadol was shown to rescue sleep in short-sleeping flies (Fig. 3.9), but did not rescue oxidative stress sensitivity in neuronal *inc-RNAi* flies (Fig. 3.10). This experiment requires further optimization to ensure that the dosage of Gaboxadol is appropriate for different genetic backgrounds. Toxicity caused by too high a dosage of Gaboxadol would be difficult to distinguish from increased sleep by an activity monitor because both result in decreased movement. Additionally, 12 hour mechanical sleep deprivation was successful in reducing sleep in only one of the two control flies tested (*elav* control), but nonetheless, did not result in any change in sensitivity to oxidative stress in any genotype (Fig.11). These challenges will be further discussed in Chapter V.

Lastly, new data have provided some interesting insight into the relationship between sleep and ROS. Several mutants harboring defects in genes that respond to oxidative stress display severely altered sleep (Fig. 3.12). However, it is unclear exactly

how ROS is affected in each of these mutants, making the interpretation of this result difficult. Additionally, flies overexpressing neuronal *SOD1* or *SOD2*, which was shown to reduce sleep in Chapter II (Fig. 2.8), are sensitive to H_2O_2 feeding (Fig. 3.13). This finding suggests that sleep loss reduces the body's ability survive oxidative stress.

Methods

Heat Stress Assay

Flies were stored in 37° or 33° C incubators throughout experiment. Vials containing different genotypes of flies were interweaved in case of heat pockets within the incubator. Death was assessed every hour (37°) or several times a day (33°) by tapping the vile vigorously and recording the number of flies that showed no movement. Flies kept at 33° were flipped onto new vials every other day.

Antioxidant Feeding

N-acetyl-cysteine was dissolved in water and added to melted cornmeal food to a concentration of 10 mM. Melatonin was dissolved in water containing 2% ethanol and added to melted cornmeal food to a concentration of 2 mM. In each case, control food was made by adding the appropriate amount of vehicle alone to melted cornmeal food.

H_2DCF Assay

Flies were decapitated on ice. 30 heads per sample were gently homogenized by hand in MIM buffer. Homogenate was subjected to a low speed centrifugation to pellet debris.

Homogenate was then centrifuged at higher speed; supernatant was removed (cytosolic fraction) and pellet was resuspended in respiration buffer (mitochondrial fraction). H_2DCF was dissolved in anhydrous DMSO, and then diluted in respiration buffer and added to the homogenate to a final concentration of 15 uM. Samples were added to a (clear bottom) black 96 well plate and fluorescence was measured by a fluorescent plate reader.

Lipid Peroxidation Assay

30 whole flies or 40 heads were used per biological replicate. MDA levels were measured following the specifications of the TBARS Assay Kit (Cayman Chemical) Colorimetric Protocol.

DHE Injection and Imaging Assay

100 nL of 20 mM DHE (dissolved in anhydrous DMSO and diluted in PBS) were injected into live flies. Flies were immediately superglued to a coverslip, with wings spread, and imaged through the dorsal cuticle of the abdomen using an epifluorescent microscope. Mean fluorescence values were obtained by tracing the first three abdominal segments below the thorax using Image J software.

Mechanical Sleep Deprivation Assay

Drosophila Activity Monitors were attached to a Troemner multi-tube vortexer that was stored inside an incubator. The vortexer was set to shake for 2 seconds at random

intervals every 2 to 36 sec. Shaking occurred for the full 12 hours of the subjective night.

Sleep Analysis, Paraquat Injection, H_2O_2 feeding, qPCR, and Gaboxadol Feeding Assay: See Chapter II Methods.

Chapter IV: The Loneliness Effect on Health

An estimated 20-40% of older people in Western nations describe themselves as lonely, and upwards of 7% report chronic loneliness [172]. A longitudinal study from 2006 found that these numbers are growing; in fact, the number of Americans who report having no close personal contacts has tripled in only two decades [173]. This increase in loneliness is especially distressing given the number of harmful effects that loneliness has been shown to have on health and general well-being. Several studies have shown that socially isolated individuals experience higher mortality rates than non-isolated individuals with similar biological and behavioral health factors [172,174]. More specifically, lonely people tend to develop both heart disease and cancer more often than others, and those who remain lonely during these illnesses tend to have worse outcomes [175]. Based on these data, social isolation has been likened to health risk factors as serious as obesity, sedentary lifestyles, and possibly even smoking [176]. One study that investigated the possible mechanisms underlying the increased mortality rate in lonely individuals identified cardiovascular activation and sleep dysfunction as two potential pre-disease mechanisms [177]; however, few studies of this kind have been reported and little progress has been made in understanding these mechanisms on a molecular level.

One effect of social isolation that has been widely studied in both humans and other mammals is immune system dysfunction. Studies conducted on first year medical students and psychiatric inpatients showed that lonelier individuals tended to have poorer cellular immunity, relative to their less lonely counterparts [178–180]. Likewise, a

study in college freshmen showed that loneliness correlated with compromised humoral immunity, indicated by decreased antibody response to an influenza vaccine [181]. Several groups have used social mammals to model the effects of social isolation on health and immunity. Social isolation has been shown to contribute to obesity and type 2 diabetes in mice [182], lower expression of glucocorticoid regulating genes in the frontal cortex of piglets [183], raise cortisol production and lower lymphocyte proliferative response to mitogens in pigs [184], increase oxidative stress in the aortic arch of rabbits [185], and heighten the morning-associated rise in cortisol production in squirrel monkeys [186]. Given the inhibitory effects of glucocorticoids, including cortisol, on the immune system, these studies provide supporting evidence that social isolation causes immune dysfunction, among other deleterious effects, in mammals.

Research has also been conducted on the effects of social isolation in invertebrates. Social isolation in the crayfish [187], cricket [188], honey bee [189], wasp [190], and fruit fly [191–193]increased aggression, as well as locomotive activity and dispersion in butterflies [194] and locusts [195]. In response to social isolation, *Drosphila* in particular has also exhibited reduced need for sleep [196], decreased fiber number in the mushroom bodies [197], and altered nerve and muscle excitability and enhanced synaptic transmission at larval neuromuscular junctions (NMJs) [198]. One group found that flies carrying mutations in two genes implicated in redox metabolism, Hyperkinetic (Hk) and glutathione S-transferase-S1 (gsts1), showed increased aggression and larval neuromuscular hyperexcitability compared to wild-type flies, even in group settings. These mutants had increased reactive oxygen species (ROS) at larval

NMJs [198]. Such findings raise the possibility that ROS may be involved in altering the behavior of socially isolated flies.

Though *Drosophila* is a social species, it does not exhibit the phenomenon of social immunity that has been described in other invertebrates. Studies in insects such as honey bees, locusts, and ants have described social immunity strategies including the following: behavioral fever, in which members of a group huddle together and move to warmer locations in order to reach a temperature that limits pathogen growth [199]; physical removal of parasites through grooming behaviors [200]; quarantine, or the removal of corpses from the nest [201]; and the incorporation of anti-microbial materials in nest building [202].

While collective defenses against disease have not been described in Drosophila, its cellular defenses against disease have been widely studied. Innate immunity in the fly is characterized by three main immune responses: anti-microbial peptide (AMP) production, melanization, and phagocytosis. Flies utilize different immune branches depending on the type of infection they are combatting.

Here, I investigated the impact of social insolation on immunity in the fruit fly. I challenged flies with a panel of different bacteria in order to analyze the function of the different immune mechanisms in response to social isolation. Significant challenges were encountered in this project, which will be discussed below.

Results

To characterize the effects of social context on immunity against infection, I conducted survival assays on male wild-type Oregon R (OR) and Canton S (CS) flies injected with

either *S. marcescens* or *L. monocytogenes*. Two social contexts were used in these experiments: complete isolation, in which a single fly was placed in a vial that had been physically separated from other vials by cardboard dividers, and group setting, in which 20 flies were placed together in a vial. In the case of *S. marcescens* infection, I found that isolated OR flies died dramatically faster than their grouped siblings in about 1/5 of the experiments conducted (Fig. 4.1A). Other times, I observed smaller and not significant trends, and some of the time I observed no difference. Similar results were obtained with CS flies (data not shown).

Interestingly, in the case of *L. monocytogenes* infection, OR flies consistently showed no difference in survival between isolated and grouped flies (Fig. 4.1B), and similar results were observed in CS flies (data not shown). Reduced survival after infection with only specific bacterial pathogens suggests that social isolation impacts only certain aspects of immune function, since different pathogens elicit different immune responses.

Discussion

S. marcescens infection elicits a phagocytic response in the fruit fly, while *L. monocytogenes* elicits a melanization response, suggesting that social isolation specifically impacts phagocytosis, but has no effect on the melanization response. However, I did not pursue further investigation with this project because I was unable to obtain consistent results with *S. marcenscens* infection or with other pathogens tested. In fact, the only consistent result I obtained was that social isolation had no impact on survival after *L. monocytogenes*.

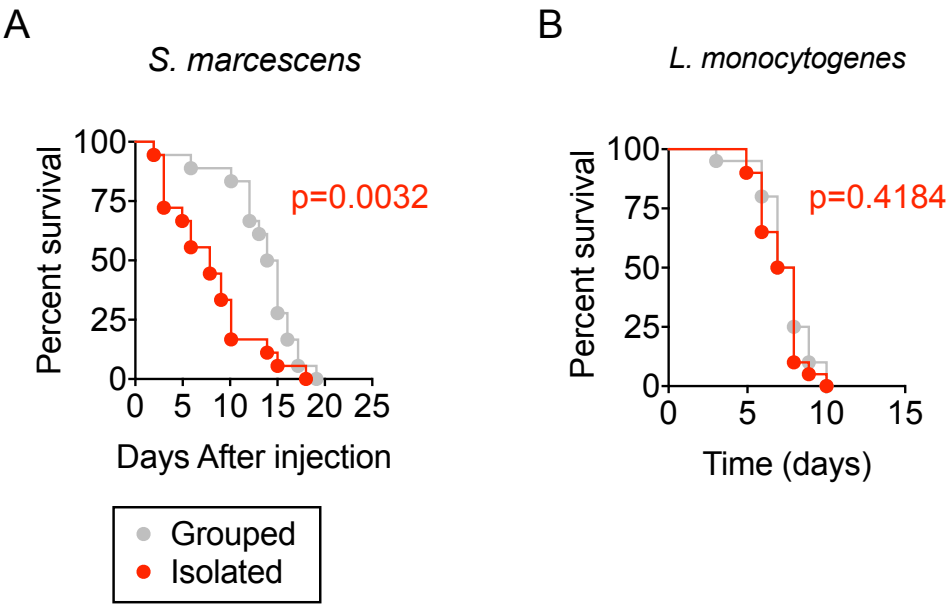

Fig. 4.1. Isolated flies are sensitive to some pathogens but not others.
Isolated OR flies died significantly faster than grouped flies after injection with *S. marcescens* (A, p=00032, n=20 flies/condition). This experiment is representative of the 1/5 experiments that were significant; others showed similar trends or no trend at all. Isolated and grouped OR flies consistently died at the same rate after injection with *L. monocytogenes* (B, p=0.0418, n=20 flies/condition). p-values were obtained by log-rank analysis.

Interestingly, sleep is reduced in the context of social isolation in fruit flies [151] and social isolation has been shown to cause increased ROS levels in the cortex of mice [203]. It would be interesting to re-visit this paradigm to test other pathogens, such as *P. rettgeri*, or to look for differences in response to oxidative stress.

Methods

Bacterial Injections: See Chapter II Methods.

Chapter V: Discussion

In Chapters II and III, short-sleeping flies were used as a model of chronic sleep restriction, representing a new approach to sleep research that diverges from the standard method of mechanical sleep deprivation. A molecularly diverse group of short-sleeping mutants share a common phenotype of oxidative stress sensitivity. Other genetic and pharmacological approaches demonstrate that increasing sleep promotes resistance to oxidative stress, and that reducing ROS levels in the brain reduces sleep. Gene expression data also indicate that ROS levels may be increased in the brains of short-sleeping mutants. This is the first evidence demonstrating a clear relationship between sleep and oxidative stress in the fruit fly.

Together, these data fit a model in which ROS levels in the brain regulate sleep, and sleep serves to clear ROS from the brain (and potentially from the body). A metabolically active brain produces ROS that accumulate over the course of the wake period, until they reach a critical threshold. An unknown mechanism (ie. ROS-activated opening of potassium channels to reduce neuronal excitability) initiates sleep, at which point the metabolic rate of the brain dramatically drops. A lower rate of ROS production provides antioxidants with a chance to neutralize the remaining ROS from neurons. This process is aided by the glymphatic system [106] which increases convective flow during sleep, thereby physically removing ROS from the brain. When ROS are lowered to a critical threshold, the same unknown mechanism (i.e. potassium channels closing to induce neuronal excitability) initiates wake.

However, several unanswered questions still remain. Without direct measurements of ROS in the brain, we can't be sure that ROS accumulates in the brains of short-sleeping flies. Also, it is unclear whether susceptibility to oxidative stress in short-sleeping flies is caused by heightened levels of ROS in the brain, or whether sleep restores antioxidant function to the body. These and other remaining questions and future directions will be discussed below.

Heat Stress and the Unfolded Protein Response

Confirmation that not all short-sleeping mutants are sensitive to heat stress provides two important insights: 1) First, this result helps to confirm that short-sleeping mutants are not simply sensitive to all forms of stress across the board. Though these mutants are all short-sleeping, they harbor mutations in diverse genes that result in varied responses to heat stress and immune challenge. Thus, their shared susceptibility to oxidative stress is unique, and illuminates how vital sleep is for this function. 2) Second, heat stress should induce protein misfolding and activate the unfolded protein response (UPR) in the ER and mitochondria. Increased expression of the ER UPR gene *BiP* in sleep-deprived flies [21,204] and rodents [149,167] has been presented as evidence that sleep serves a vital role in clearing misfolded proteins. Because high levels of ROS can also induce protein misfolding and activation of the UPR, it is important to distinguish between the two types of stress. Lack of a global heat sensitivity across short-sleeping mutants argues against clearance of misfolded proteins as the primary function of sleep.

This is further supported by our gene expression data in neuronal *inc-RNAi* flies, in which antioxidant genes are strongly induced at baseline, whereas *BiP* shows just a slight and not statistically significant increase (Fig. 2.7). However, the ER UPR has been shown to activate the ROS sensor and antioxidant transcription factor *nrf2* downstream of *Bip* [205]. Additionally, we did observe increased baseline expression of two mitochondrial UPR genes (Fig. 2.7). Though induction of mitochondrial stress genes is expected in the context of high baseline ROS levels, which are particularly damaging to mitochondria, exploring the expression of additional antioxidant genes that are not under the control of *nrf2* may be helpful in clarifying whether ROS-specific stress responses precede UPR activation in the context of sleep deprivation.

It is likely that sleep serves an important function in protecting against both high ROS and protein misfolding, and these functions are not mutually exclusive, since changes in ROS can cause protein misfolding. While I demonstrated in Chapters II and III that manipulating ROS levels in the fly directly effects baseline sleep levels (Fig. 2.8, 3.12), it has been shown that *BiP* mutants have normal levels of baseline sleep but a defect in recovery sleep following sleep deprivation [204]. Thus, the two types of stress may control different types of sleep homeostasis: for instance, daily fluctuations in ROS levels may control baseline sleep, while protein misfolding, the downstream effect of ROS accumulation, may trigger recovery sleep after sleep deprivation.

Gene Expression

Baseline induction of antioxidant and mitochondrial stress response genes in the heads of neuronal *inc-RNAi* flies provides indirect evidence that these flies have elevated

baseline neuronal ROS levels. However, verification in other short-sleeping mutants is necessary, and confirmation by direct measurement of ROS in the brain, such as by DHE staining of brain tissue, would be ideal.

The gene expression pattern of neuronal *inc-RNAi* flies following paraquat injection provides additional insight into their susceptibility to oxidative challenge. The failure of neuronal *inc-RNAi* flies to induce *catalase* and *hsp60* as well as their reduced expression of *Pink1* and *ClpX* in the head in response to paraquat (Fig. 3.5 E-F) suggests that they may be redirecting to cell death pathways, rather than continuing induction of stress response pathways. Indeed, prolonged activation of the UPR has been shown to induce apoptosis [206]. It would be interesting to investigate the expression of apoptotic genes in the context of paraquat injection.

Lastly, it would be interesting to compare gene expression in the head with gene expression in the body, especially since both ROS-inducing agents used to induce oxidative stress in Chapters II and III were administered through the body (paraquat injection into the abdomen and H_2O_2 feeding). Preliminary comparison of body and head expression has revealed a difference in *catalase*: *catalase* expression in the head is not induced at baseline (Fig. 2.7), but it is upregulated in the body compared to controls (data not shown). Additional measurements of gene expression in the body may also help to elucidate the cause of death in short-sleeping flies following paraquat injection. This information would be particularly interesting in light of the more recent data from flies overexpressing neuronal *SOD1* and *SOD2*, which I found to be sensitive to H_2O_2 feeding. This result suggests that ROS levels in the brain do not directly

influence the outcome of exposure of the whole body to ROS, and instead indicate that antioxidant response in the body may have a larger impact on survival.

Antioxidant Feeding

Similar to the reduction of sleep observed in flies overexpressing antioxidants in neurons, I showed in Chapter III that feeding wild type flies antioxidants also reduces sleep. However, the sleep reduction by antioxidant feeding was more subtle than by neuron-specific overexpression of antioxidants. This is likely because antioxidants need to reach the brain in order to have an effect on sleep. Because this assay involves the comparison of antioxidant-fed and vehicle-fed flies, it is also important to conduct a CAFÉ assay to verify that both groups ate comparable amounts of food throughout the experiment. Drugs can be bitter and if flies avoid drug-laced food, starvation can induce wakefulness, creating the appearance of a drug-induced effect. This is a problem that has been addressed in the context of caffeine feeding in flies [207]. If it can be confirmed that flies eat antioxidant-laced food as much as control food, then this result supports the conclusion that ROS levels in the brain regulate sleep.

Direct Measurement of ROS or Oxidative Damage

I encountered considerable obstacles in each of the methods that I used to measure ROS levels. Initially, adding H_2DCF to fly homogenate and measuring fluorescence in a plate reader assay yielded very variable results. I found that splitting the homogenate into cytosolic and mitochondrial fractions produced more consistent results, though differences were always small and often not significant. I did see a consistent trend of

increased H_2DCF signal in heads from neuronal *inc-RNAi* flies compared to controls in the cytosolic fraction. However, I also consistently saw that neuronal *inc-RNAi* flies had lower H_2DCF signal compared to the *inc-RNAi* control, and comparable levels compared to the *elav* control, in the mitochondrial fraction. It is difficult to interpret this difference between fractions. One explanation is that the cytosolic fraction reflects the overall ROS levels in the head directly following death; superoxide produced from the mitochondria is quickly converted to diffusible H_2O_2 which likely enters the cytosol. Since this assay is conducted in respiration buffer which provides live mitochondria with metabolic substrates, and ROS is a byproduct of respiration, the fluorescence levels from the mitochondrial fraction may instead reflect active respiration in the mitochondria isolated from the fly homogenate. In this case, reduced fluorescence in the mitochondrial fraction could indicate that neuronal *inc-RNAi* flies have damaged mitochondria that are not able to respire at the same metabolic rate as controls. While the results from the H_2DCF assay do add some support to the hypothesis that short-sleeping flies have elevated neuronal ROS, evidence from other methods of ROS measurement are necessary to verify this hypothesis.

As a less direct proxy for ROS levels, I measured lipid peroxidation, which is a commonly measured form of oxidative damage. Results from this assay indicated no difference in lipid peroxidation in the whole body or head of neuronal *inc-RNAi* flies compared to controls. This result indicates that, if ROS levels are high in neuronal *inc-RNAi* brains, they are not so high as to induce oxidative damage. This is not surprising, given that excessive oxidative damage in the brain would cause shortened lifespan, which we don't see in neuronal *inc-RNAi* flies (Fig. 2.1). If ROS does play a role in

regulating sleep, ROS levels likely fluctuate within a very narrow range in order to induce sleep without inducing oxidative damage, which cannot be reversed. It would be interesting to repeat this assay in other short-sleeping mutants—especially mutants that have a shortened lifespan that may be caused by oxidative damage in the brain.

Finally, I measured ROS levels directly in the body using a DHE injection assay. This assay was particularly challenging for several reasons: 1) DHE easily crystalizes, which can clog the injection needle, 2) this assay requires large injection volumes, which often cause the flies to burst, 3) variability in mean fluorescence between individual flies is very high. Despite these differences, I did observe a very slight trend toward increased ROS in neuronal *inc-RNAi* flies, and a more noticeable increase in *fumin* flies. However, in both cases, injection with the control dye Alexa568, which has a similar emission wavelength to DHE, also resulted in increased fluorescence. This difference could not be explained by reduced size in the mutants, since I found *fumin* mutants to actually be slightly larger than controls. Another possibility is that Alexa568 is also sensitive to oxidation. Perhaps a better use of DHE would be to stain the brain and other tissues of short-sleeping mutants to determine if ROS levels are high.

Overall, the only consistent and reliable difference that I observed was an increase in ROS, as indicated by increased fluorescence of the ROS-sensitive dye H_2DCF, in the cytosolic fraction of head homogenate from neuronal *inc-RNAi* flies relative to controls. The data from all three methods used to measure ROS or oxidative damage are summarized in Figure 5.1.

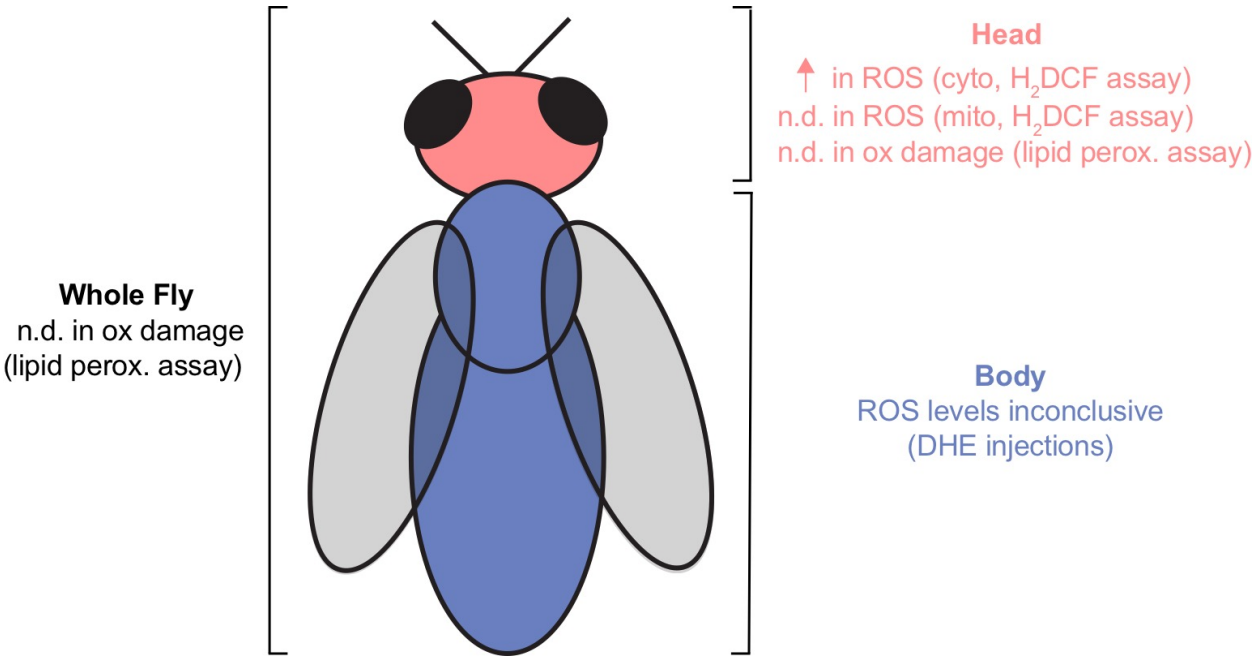

Fig. 5.1. Summary of ROS levels and oxidative damage in neuronal *inc-RNAi* flies. Three methods were used to measure ROS levels or oxidative damage: (1) H_2DCF assay, (2) DHE injection, or (3) lipid peroxidation assay. (1) To measure ROS levels directly in the head, fly homogenate from heads was treated with the ROS-sensitive dye H_2DCF and fluorescence was measured in a plate reader assay. Homogenate was separated into a cytosolic fraction or a mitochondrial fraction. Significantly increased fluorescence in the cytosolic fraction relative to controls indicated increased ROS in the heads of neuronal *inc-RNAi* flies. Fluorescence in the mitochondrial fraction was comparable to one of the two controls (*elav* control). (2) To measure ROS levels in the body, live flies were injected with the ROS-sensitive dye DHE and imaged through the dorsal cuticle using an epifluorescent microscope to obtain mean fluorescence values. While there was a significant increase in fluorescence in neuronal *inc-RNAi* flies compared to controls, this was also the case when flies were injected with the control dye Alexa 568, indicating that the difference in DHE fluorescence was an artifact. Thus, the data from the DHE injections was inconclusive. (3) To measure oxidative damage in the whole fly or head, a lipid peroxidation assay was performed. There were no differences in lipid peroxide levels in the whole fly or in the head of neuronal *inc-RNAi* flies compared to controls, indicating no differences in oxidative damage.
n.d. = no difference; cyto = cytosolic fraction; mito = mitochondrial fraction.

Rescuing sleep in short-sleeping mutants

In Chapter III, I fed Gaboxadol to neuronal *inc-RNAi* flies in order to induce sleep, and tested for rescued oxidative stress sensitivity (Fig. 3.10). However, I found that Gaboxadol fed controls died faster after paraquat injection, which contradicts my earlier result that Gaboxadol-fed wild type flies survived longer after paraquat injection (Fig. 2.6). Given that the same concentration of Gaboxadol resulted in very different degrees of sleep induction in short-sleeping mutants (Fig. 9), I suspect that this particular dosage of Gaboxadol was toxic to neuronal *inc-RNAi* flies and controls. Data from an activity monitor cannot distinguish between flies that are moving less due to increased sleep and flies that are moving less due to drug overdose. A better approach to this question may be to induce sleep genetically in short sleeping mutants using a FB driver and the sodium bacterial channel construct *NaChBac,* and then test for rescued oxidative stress response.

Mechanical Sleep Deprivation

While the result obtained from mechanical sleep deprivation in neuronal *inc-RNAi* and control flies suggests that sleep deprivation does not induce oxidative stress sensitivity, this experiment posed several challenges. First, paraquat injection and H_2O_2 feeding take days to kill flies, but I was unable to sleep deprive flies past 12 hours without them showing rebound sleep during shaking. Thus, sleep-deprivation was not occurring throughout the entire course of oxidative challenge. Also, once flies are removed from sleep-deprivation, a sleep rebound occurs. It has been reported that *inc* null mutants do not exhibit a sleep rebound following mechanical deprivation [44], but it would be

interesting to analyze the sleep data from these experiments following sleep deprivation to determine whether neuronal *inc-RNAi* flies are capable of normal rebound. If they are not able to rebound properly, then this would put them at an additional disadvantage in this experimental setup, and we would no longer be comparing flies with equal levels of sleep. One way to potentially solve these issues would be to increase the dosage of paraquat such that death occurs within the 12 hour period of sleep deprivation. Another factor to consider in the experimental setup is whether to sleep deprive flies before paraquat injection, rather than during.

Reduced Sleep in Stress Response Mutants

In Chapter III, I presented sleep data from flies with mutations in genes that respond to high ROS levels (Fig. 3.12). The sleep reduction and corresponding increase in bout number were striking in similarity between these mutants. This result supports a link between sleep and oxidative stress, but is confusing for a number of reasons. These mutants were initially selected because I expected them to have high levels of ROS, and was testing to see if increased ROS would result in increased sleep. However, none of these mutants have been characterized previously and oxidative stress assays yielded variable results depending on the concentration of oxidizing agent (Appendix III, Table 3.2). Because there are redundancies in stress pathways, it is possible that these mutants exhibit compensatory inductions of other stress response genes that complicate their response to oxidative stress. Alternatively, high ROS levels in the body may initiate a stress response that induces wake as an evolutionary adaptation to encourage the animal to move away from the noxious agent. A much simpler approach

to this question would be to drive expression of RNAi against antioxidants in the brain or in the whole body and measure sleep levels.

Effects of Neuronal Antioxidant Overexpression

Based on the logic that neuronal ROS accumulation in short-sleeping mutants may cause their reduced survival after oxidative challenge, I expected flies with neuronal overexpression of antioxidants to be resistant to oxidative stress. However, I found that overexpression of *SOD1* and *SOD2* caused reduced survival after oxidative challenge (3.13). One explanation of this result is that, as shown in Chapter II, reduced ROS levels in the brain decrease sleep, thereby increasing the fly's sensitivity to oxidative stress. In this case, sensitivity to oxidative stress in short-sleeping mutants would be caused by a defect in the body's inability to neutralize ROS, rather than the brain's sensitivity to oxidative stress. Follow up with gene expression data in the body of short-sleeping mutants, as well as DHE staining for tissues such as the gut, may help to elucidate this finding.

Conclusion

Though further investigation is necessary to answer these remaining questions, this thesis demonstrates, for the first time in *Drosophila,* a clear relationship between sleep and oxidative stress. The idea that sleep clears ROS from the brain was proposed over two decades ago, but never substantiated due to conflicting data in sleep deprived rats. The genetic tools now available to manipulate sleep in fruit flies offer a powerful new approach to these studies. Modern society is plagued by chronic sleep restriction, which

is associated with various poor health outcomes; thus, a better understanding of the biology of sleep is crucial. Using short-sleeping flies as a model for chronic sleep restriction has provided valuable insight into the age-old question of why we sleep.

References

1. Lavie P. The Enchanted World of Sleep. New Haven, CT: Yale University Press; 1998.
2. MacNish R. Philosophy of Sleep. Glasgow: Curll & Bell, printers; 1830.
3. K Ishimori. True cause of sleep: a hypnogenic substance as evidenced in the brain of sleep-deprived animals. Tokyo Igakkai Zasshi. 1909;23: 429–457.
4. Legendre R, Pieron H. Recherches sur le besoin de sommeil consecutif a une veille prolongee. Z Allgem Physiol. 1913;14: 235–262.
5. Jean-Louis G, Kripke DF, Ancoli-Israel S, Klauber MR, Sepulveda RS. Sleep duration, illumination, and activity patterns in a population sample: effects of gender and ethnicity. Biol Psychiatry. 2000;47: 921–927. doi:10.1016/S0006-3223(99)00169-9
6. Johnson NB, Hayes LD, Brown K, Hoo EC EKC for DC and P (CDC). CDC National Health Report: leading causes of morbidity and mortality and associated behavioral risk and protective factors--United States, 2005-2013. MMWR Suppl. 2014;63: 3–27.
7. Nedeltcheva A V, Scheer FAJL. Metabolic effects of sleep disruption, links to obesity and diabetes. Curr Opin Endocrinol Diabetes Obes. 2014;21: 293–8. doi:10.1097/MED.0000000000000082
8. Cappuccio FP, Cooper D, Delia L, Strazzullo P, Miller MA. Sleep duration predicts cardiovascular outcomes: A systematic review and meta-analysis of prospective studies. European Heart Journal. 2011. pp. 1484–1492.

doi:10.1093/eurheartj/ehr007

9. Lim ASP, Kowgier M, Yu L, Buchman AS, Bennett DA. Sleep Fragmentation and the Risk of Incident Alzheimer's Disease and Cognitive Decline in Older Persons. Sleep. 2013;36: 1027–1032. doi:10.5665/sleep.2802
10. Cirelli C. The genetic and molecular regulation of sleep: From fruit flies to humans. Nat Rev Neurosci. Nature Publishing Group; 2009;10: 549–560. doi:10.1038/nrn2683
11. Campbell SS, Tobler I. Animal sleep: A review of sleep duration across phylogeny. Neurosci Biobehav Rev. 1984;8: 269–300. doi:10.1016/0149-7634(84)90054-X
12. Hendricks JC, Sehgal A, Pack AI. The need for a simple animal model to understand sleep. Prog Neurobiol. 2000;61: 339–351. doi:10.1016/S0301-0082(99)00048-9
13. Kaiser W, Steiner-Kaiser J. Neuronal correlates of sleep, wakefulness and arousal in a diurnal insect. Nature. 1983;301: 707–709. doi:10.1038/301707a0
14. Tobler I, Stalder J. Rest in the scorpion - a sleep-like state? J Comp Physiol A. 1988;163: 227–235. doi:10.1007/BF00612431
15. Mendoza-Angeles K, Cabrera A, Hernández-Falcón J, Ramón F. Slow waves during sleep in crayfish: A time-frequency analysis. J Neurosci Methods. 2007;162: 264–271. doi:10.1016/j.jneumeth.2007.01.025
16. Nitz DA, Van Swinderen B, Tononi G, Greenspan RJ. Electrophysiological correlates of rest and activity in Drosophila melanogaster. Curr Biol. 2002;12: 1934–1940. doi:10.1016/S0960-9822(02)01300-3

17. Eban-Rothschild AD, Bloch G. Differences in the sleep architecture of forager and young honeybees (Apis mellifera). J Exp Biol. 2008;211: 2408–2416. doi:10.1242/jeb.016915

18. van Alphen B, Yap MHW, Kirszenblat L, Kottler B, van Swinderen B. A Dynamic Deep Sleep Stage in Drosophila. J Neurosci. 2013;33: 6917–6927. doi:10.1523/JNEUROSCI.0061-13.2013

19. Yokogawa T, Marin W, Faraco J, Pézeron G, Appelbaum L, Zhang J, et al. Characterization of sleep in zebrafish and insomnia in hypocretin receptor mutants. PLoS Biol. 2007;5: 2379–2397. doi:10.1371/journal.pbio.0050277

20. Hendricks JC, Finn SM, Panckeri KA, Chavkin J, Williams JA, Sehgal A, et al. Rest in Drosophila Is a Sleep-like State. Neuron. 2000;25: 129–138. doi:10.1016/S0896-6273(00)80877-6

21. Shaw PJ. Correlates of Sleep and Waking in Drosophila melanogaster. Science (80-). 2000;287: 1834–1837. doi:10.1126/science.287.5459.1834

22. Raizen DM, Zimmerman JE, Maycock MH, Ta UD, You YJ, Sundaram M V., et al. Lethargus is a Caenorhabditis elegans sleep-like state. Nature. 2008;451: 569–572. doi:10.1038/nature06535

23. Siegel JM. Do all animals sleep? Trends Neurosci. 2008;31: 208–213. doi:10.1016/j.tins.2008.02.001

24. Hobson J. Electrographic correlates of behavior in the frog with special reference to sleep. Electroencephalogr Clin Neurophysiol. 1967;22: 113–21. doi:10.1016/0013-4694(67)90150-2

25. Goldshmid R, Holzman R, Weihs D, Genin A. Aeration of corals by sleep-

swimming fish. Limnol Oceanogr. 2004;49: 1832–1839. doi:10.4319/lo.2004.49.5.1832

26. Holbrook SJ, Schmitt RJ. Competition for shelter space causes density-dependent predation mortality in damselfishes. Ecology. 2002;83: 2855–2868. doi:10.1890/0012-9658(2002)083[2855:CFSSCD]2.0.CO;2

27. Ridgway S. Dolphin continuous auditory vigilance for five days. J Exp Biol. 2006;209: 3621–3628. doi:10.1242/jeb.02405

28. Cirelli C, Tononi G. Is sleep essential? PLoS Biol. 2008;6: 1605–1611. doi:10.1371/journal.pbio.0060216

29. Rattenborg NC, Amlaner CJ, Lima SL. Behavioral, neurophysiological and evolutionary perspectives on unihemispheric sleep. Neuroscience and Biobehavioral Reviews. 2000. pp. 817–842. doi:10.1016/S0149-7634(00)00039-7

30. Libourel P-A, Herrel A. Sleep in amphibians and reptiles: a review and a preliminary analysis of evolutionary patterns. Biol Rev. 2016;91: 833–866. doi:10.1111/brv.12197

31. Hendricks JC, Kirk D, Panckeri K, Miller MS, Pack AI. Modafinil maintains waking in the fruit fly drosophila melanogaster. Sleep. 2003;26: 139–146. Available: http://cat.inist.fr/?aModele=afficheN&cpsidt=14643562

32. Dissel S, Angadi V, Kirszenblat L, Suzuki Y, Donlea J, Klose M, et al. Sleep restores behavioral plasticity to drosophila mutants. Curr Biol. Elsevier Ltd; 2015;25: 1270–1281. doi:10.1016/j.cub.2015.03.027

33. Berry JA, Cervantes-Sandoval I, Chakraborty M, Davis RL. Sleep Facilitates Memory by Blocking Dopamine Neuron-Mediated Forgetting. Cell. Elsevier Inc.;

2015;161: 1656–1667. doi:10.1016/j.cell.2015.05.027

34. Cirelli C, Bushey D, Hill S, Huber R, Kreber R, Ganetzky B, et al. Reduced sleep in Drosophila Shaker mutants. Nature. 2005;434: 1087–1092. doi:10.1038/nature03486

35. Bushey D, Huber R, Tononi G, Cirelli C. Drosophila Hyperkinetic Mutants Have Reduced Sleep and Impaired Memory. J Neurosci. 2007;27: 5384–5393. doi:10.1523/JNEUROSCI.0108-07.2007

36. Koh K, Joiner WJ, Wu MN, Yue Z, Smith CJ, Sehgal A. Identification of SLEEPLESS, a Sleep-Promoting Factor. Science (80-). 2008;321: 372–376. doi:10.1126/science.1155942

37. Wu M, Robinson JE, Joiner WJ. SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission. Curr Biol. Elsevier Ltd; 2014;24: 621–629. doi:10.1016/j.cub.2014.02.026

38. Kume K. Dopamine Is a Regulator of Arousal in the Fruit Fly. J Neurosci. 2005;25: 7377–7384. doi:10.1523/JNEUROSCI.2048-05.2005

39. Donlea JM, Thimgan MS, Suzuki Y, Gottschalk L, Shaw PJ. Inducing Sleep by Remote Control Facilitates Memory Consolidation in Drosophila. Science (80-). 2011;332: 1571–1576. doi:10.1126/science.1202249

40. Ueno T, Tomita J, Tanimoto H, Endo K, Ito K, Kume S, et al. Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. Nat Neurosci. 2012;15: 1516–1523. doi:10.1038/nn.3238

41. Shi M, Yue Z, Kuryatov A, Lindstrom JM, Sehgal A. Identification of Redeye, a new sleepregulating protein whose expression is modulated by sleep amount.

Elife. 2014;2014: 1–17. doi:10.7554/eLife.01473

42. Platt B, Riedel G. The cholinergic system, EEG and sleep. Behavioural Brain Research. 2011. pp. 499–504. doi:10.1016/j.bbr.2011.01.017

43. Stavropoulos N, Young MW. Insomniac and cullin-3 regulate sleep and wakefulness in drosophila. Neuron. Elsevier Inc.; 2011;72: 964–976. doi:10.1016/j.neuron.2011.12.003

44. Pfeiffenberger C, Allada R. Cul3 and the BTB Adaptor Insomniac Are Key Regulators of Sleep Homeostasis and a Dopamine Arousal Pathway in Drosophila. PLoS Genet. 2012;8. doi:10.1371/journal.pgen.1003003

45. Konopka RJ, Benzer S. Clock mutants of Drosophila melanogaster. Proc Natl Acad Sci U S A. 1971;68: 2112–6. doi:10.1073/pnas.68.9.2112

46. Allada R, Chung BY. Circadian organization of behavior and physiology in Drosophila. Annu Rev Physiol. 2010;72: 605–24. doi:10.1146/annurev-physiol-021909-135815

47. Hardin PE, Panda S. Circadian timekeeping and output mechanisms in animals. Curr Opin Neurobiol. Elsevier Ltd; 2013;23: 724–731. doi:10.1016/j.conb.2013.02.018

48. Renn SCP, Park JH, Rosbash M, Hall JC, Taghert PH. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell. 1999;99: 791–802. doi:10.1016/S0092-8674(00)81676-1

49. Aton SJ, Colwell CS, Harmar AJ, Waschek J, Herzog ED. Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock

neurons. Nat Neurosci. 2005;8: 476–483. doi:10.1038/nn1419

50. Toh KL. An hPer2 Phosphorylation Site Mutation in Familial Advanced Sleep Phase Syndrome. Science (80-). 2001;291: 1040–1043. doi:10.1126/science.1057499

51. Archer SN, Robilliard DL, Skene DJ, Smits M, Williams A, Arendt J, et al. A Length Polymorphism in the Circadian Clock Gene Per3 is Linked to Delayed Sleep Phase Syndrome and Extreme Diurnal Preference. Sleep. 2003;26: 413–415. doi:10.1093/sleep/26.4.413

52. Carpen JD, Von Schantz M, Smits M, Skene DJ, Archer SN. A silent polymorphism in the PER1 gene associates with extreme diurnal preference in humans. J Hum Genet. 2006;51: 1122–1125. doi:10.1007/s10038-006-0060-y

53. Eastman CI, Mistlberger RE, Rechtschaffen A. Suprachiasmatic nuclei lesions eliminate circadian temperature and sleep rhythms in the rat. Physiol Behav. 1984;32: 357–368. doi:10.1016/0031-9384(84)90248-8

54. Shiromani PJ, Xu M, Winston EM, Shiromani SN, Gerashchenko D, Weaver DR. Sleep rhythmicity and homeostasis in mice with targeted disruption of mPeriod genes. Am J Physiol Regul Integr Comp Physiol. 2004;287: R47-57. doi:10.1152/ajpregu.00138.2004

55. Chung BY, Kilman VL, Keath JR, Pitman JL, Allada R. The GABAA Receptor RDL Acts in Peptidergic PDF Neurons to Promote Sleep in Drosophila. Curr Biol. 2009;19: 386–390. doi:10.1016/j.cub.2009.01.040

56. Sheeba V, Fogle KJ, Kaneko M, Rashid S, Chou YT, Sharma VK, et al. Large Ventral Lateral Neurons Modulate Arousal and Sleep in Drosophila. Curr Biol.

2008;18: 1537–1545. doi:10.1016/j.cub.2008.08.033

57. Kunst M, Hughes ME, Raccuglia D, Felix M, Li M, Barnett G, et al. Calcitonin gene-related peptide neurons mediate sleep-specific circadian output in Drosophila. Curr Biol. 2014;24: 2652–2664. doi:10.1016/j.cub.2014.09.077

58. Liu S, Lamaze A, Liu Q, Tabuchi M, Yang Y, Fowler M, et al. WIDE AWAKE mediates the circadian timing of sleep onset. Neuron. Elsevier Inc.; 2014;82: 151–166. doi:10.1016/j.neuron.2014.01.040

59. Edgar DM, Dement WC, Fuller CA. Effect of SCN lesions on sleep in squirrel monkeys: evidence for opponent processes in sleep-wake regulation. J Neurosci. 1993;13: 1065–1079.

60. Easton A, Meerlo P, Bergmann B, Turek FW. The suprachiasmatic nucleus regulates sleep timing and amount in mice. Sleep. 2004;27: 1307–1318. doi:10.1093/sleep/27.7.1307

61. Hendricks JC, Lu S, Kume K, Yin JC-P, Yang Z, Sehgal A. Gender Dimorphism in the Role of cycle (BMAL1) in Rest, Rest Regulation, and Longevity in Drosophila melanogaster. J Biol Rhythms. 2003;18: 12–25. doi:10.1177/0748730402239673

62. Naylor E, Bergmann BM, Krauski K, Zee PC, Takahashi JS, Vitaterna MH, et al. The circadian clock mutation alters sleep homeostasis in the mouse. J Neurosci. 2000;20: 8138–43. doi:20/21/8138 [pii]

63. Wisor JP, O'Hara BF, Terao A, Selby CP, Kilduff TS, Sancar A, et al. A role for cryptochromes in sleep regulation. BMC Neurosci. 2002;3: 1. doi:10.1186/1471-2202-3-20

64. Shaw PJ, Tononi G, Greenspan RJ, Robinson DF. Stress response genes protect

against lethal effects of sleep deprivation in Drosophila. Nature. 2002;417: 287–291. doi:10.1038/417287a

65. Borbely AA. A two process model of sleep regulation. Human Neurobiology. 1982. pp. 195–204. doi:10.1111/jsr.12371

66. Cedernaes J, Osler ME, Voisin S, Broman JE, Vogel H, Dickson SL, et al. Acute sleep loss induces tissue-specific epigenetic and transcriptional alterations to circadian clock genes in men. J Clin Endocrinol Metab. 2015;100: E1255–E1261. doi:10.1210/JC.2015-2284

67. Meerlo P, Benca RM. Sleep, Neuronal Plasticity and Brain Function [Internet]. 2015. doi:10.1007/978-3-662-46878-4

68. Blake, H.; Gerard RW. Brain potentials during sleep. Am J Physiol. 1937;119: 692–703.

69. Vyazovskiy V V., Tobler I. Theta activity in the waking EEG is a marker of sleep propensity in the rat. Brain Res. 2005;1050: 64–71. doi:10.1016/j.brainres.2005.05.022

70. Porkka-Heiskanen T, Strecker RE, Thakkar M, Bjørkum AA, Greene RW, McCarley RW. Adenosine: A mediator of the sleep-inducing effects of prolonged wakefulness. Science (80-). 1997;276: 1265–1267. doi:10.1126/science.276.5316.1265

71. Gass N, Porkka-Heiskanen T, Kalinchuk A V. The role of the basal forebrain adenosine receptors in sleep homeostasis. Neuroreport. 2009;20: 1013–1018. doi:10.1097/WNR.0b013e32832d5859

72. Kalinchuk A V., Urrila AS, Alanko L, Heiskanen S, Wigren HK, Suomela M, et al.

Local energy depletion in the basal forebrain increases sleep. Eur J Neurosci. 2003;17: 863–869. doi:10.1046/j.1460-9568.2003.02532.x

73. Kostin A, Stenberg D, Porkka-Heiskanen T. Effect of sleep deprivation on multi-unit discharge activity of basal forebrain: Sleep and basal forebrain. J Sleep Res. 2010;19: 269–279. doi:10.1111/j.1365-2869.2009.00791.x

74. Vyazovskiy V V., Olcese U, Lazimy YM, Faraguna U, Esser SK, Williams JC, et al. Cortical Firing and Sleep Homeostasis. Neuron. 2009;63: 865–878. doi:10.1016/j.neuron.2009.08.024

75. Kalinchuk A V., McCarley RW, Porkka-Heiskanen T, Basheer R. The time course of adenosine, nitric oxide (NO) and inducible NO synthase changes in the brain with sleep loss and their role in the non-rapid eye movement sleep homeostatic cascade. J Neurochem. 2011;116: 260–272. doi:10.1111/j.1471-4159.2010.07100.x

76. Kalinchuk A V., Lu Y, Stenberg D, Rosenberg PA, Porkka-Heiskanen T. Nitric oxide production in the basal forebrain is required for recovery sleep. J Neurochem. 2006;99: 483–498. doi:10.1111/j.1471-4159.2006.04077.x

77. Porkka-Heiskanen T. Sleep homeostasis. Curr Opin Neurobiol. Elsevier Ltd; 2013;23: 799–805. doi:10.1016/j.conb.2013.02.010

78. Kalinchuk A V., Stenberg D, Rosenberg PA, Porkka-Heiskanen T. Inducible and neuronal nitric oxide synthases (NOS) have complementary roles in recovery sleep induction. Eur J Neurosci. 2006;24: 1443–1456. doi:10.1111/j.1460-9568.2006.05019.x

79. Cirelli C, Gutierrez CM, Tononi G. Extensive and Divergent Effects of Sleep and

Wakefulness on Brain Gene Expression. Neuron. 2004;41: 35–43. doi:10.1016/S0896-6273(03)00814-6

80. Cirelli C, Faraguna U, Tononi G. Changes in brain gene expression after long-term sleep deprivation. J Neurochem. 2006;98: 1632–1645. doi:10.1111/j.1471-4159.2006.04058.x

81. Williams JA, Sathyanarayanan S, Hendricks JC, Sehgal A. Interaction between sleep and the immune response in Drosophila: A role for the NF kappa B relish. Sleep. 2007;30: 389–400.

82. Hart B. Biological basis of hte behavior of sick animals. Neurosci Biobehav Rev. 1988;12: 123–137. doi:10.1016/S0149-7634(88)80004-6

83. Kuo T-H, Pike DH, Beizaeipour Z, Williams J a. Sleep triggered by an immune response in Drosophila is regulated by the circadian clock and requires the NFkappaB Relish. BMC Neurosci. 2010;11: 17. doi:10.1186/1471-2202-11-17

84. Opp MR. Cytokines and Sleep. Sleep Med Rev. 2005;9: 355–364. doi:10.1016/S1567-7443(07)10011-9

85. Krueger JM, Obál FJ, Fang J, Kubota T, Taishi P. The role of cytokines in physiological sleep regulation. Ann N Y Acad Sci. 2001;933: 211–21. doi:10.1111/j.1749-6632.2001.tb05826.x

86. Imeri L, Opp MR. How (and why) the immune system makes us sleep. Nat Rev Neurosci. 2009;10: 199–210. doi:10.1038/nrn2576

87. Peigneux P, Laureys S, Delbeuck X, Maquet P. Sleeping brain, learning brain. The role of sleep for memory systems. Neuroreport. 2001;12: A111–A124. doi:10.1097/00001756-200112210-00001

88. Gilestro GF, Tononi G, Cirelli C. Widespread changes in synaptic markers as a function of sleep and wakefulness in drosophila. Science (80-). 2009;324: 109–112. doi:10.1126/science.1166673

89. Tononi G, Cirelli C. Sleep and synaptic homeostasis: A hypothesis. Brain Research Bulletin. 2003. pp. 143–150. doi:10.1016/j.brainresbull.2003.09.004

90. Bushey D, Cirelli C. From genetics to structure to function: Exploring sleep in drosophila [Internet]. International Review of Neurobiology. Elsevier Inc.; 2011. doi:10.1016/B978-0-12-387003-2.00009-4

91. Tononi G, Cirelli C. Sleep and the Price of Plasticity: From Synaptic and Cellular Homeostasis to Memory Consolidation and Integration. Neuron. 2014. pp. 12–34. doi:10.1016/j.neuron.2013.12.025

92. Bushey D, Tononi G, Cirelli C. Sleep and Synaptic Homeostasis: Structural Evidence in Drosophila. Science (80-). 2011;332: 1576–1581. doi:10.1126/science.1202839

93. Frank MG. Sleep and synaptic plasticity in the developing and adult brain. Curr Top Behav Neurosci. 2015;25: 123–149. doi:10.1007/7854_2014_305

94. Clark DD SL. Basic Neurochemistry: Molecular, Cellular and Medical Aspects. Siegel, G. J., Agranoff, B. W., Albers, R. W., Fisher, S. K. & Uhler MD, editor. Lippincott, Philadelphia; 1999.

95. Kennedy C, Gillin JC, Mendelson W, Suda S, Miyaoka M, Ito M, et al. Local cerebral glucose utilization in non-rapid eye movement sleep. Nature. 1982;297: 325–327. doi:10.1038/297325a0

96. Heiss WD, Pawlik G, Herholz K, Wagner R, Wienhard K. Regional cerebral

glucose metabolism in man during wakefulness, sleep, and dreaming. Brain Res. 1985;327: 362–366. doi:10.1016/0006-8993(85)91537-9

97. Buchsbaum MS, Gillin JC, Wu J, Hazlett E, Sicotte N, Dupont RM, et al. Regional cerebral glucose metabolic rate in human sleep assessed by positron emission tomography. Life Sci. 1989;45: 1349–1356. Available: http://www.ncbi.nlm.nih.gov/pubmed/2796606

98. Biston P, Van Cauter E, Ofek G, Linkowski P, Polonsky KS, Degaute JP. Diurnal variations in cardiovascular function and glucose regulation in normotensive humans. Hypertension. 1996;28: 863–871. doi:10.1161/01.HYP.28.5.863

99. Zimmerman JE, Mackiewicz M, Galante RJ, Zhang L, Cater J, Zoh C, et al. Glycogen in the brain of Drosophila melanogaster: diurnal rhythm and the effect of rest deprivation. J Neurochem. 2004;88: 32–40. doi:10.1046/j.1471-4159.2003.02126.x

100. Kong J, Shepel PN, Holden CP, Mackiewicz M, Pack AI, Geiger JD. Brain glycogen decreases with increased periods of wakefulness: implications for homeostatic drive to sleep. J Neurosci. 2002;22: 5581–7. doi:20026500

101. Karnovsky ML, Reich P, Anchors JM, Burrows BL. Changes in Brain Glycogen During Slow-Wave Sleep in the Rat. J Neurochem. 1983;41: 1498–1501. doi:10.1111/j.1471-4159.1983.tb00853.x

102. Gip P, Hagiwara G, Ruby NF, Heller HC. Sleep deprivation decreases glycogen in the cerebellum but not in the cortex of young rats. Am J Physiol - Regul Integr Comp Physiol. 2002;283: R54–R59. doi:10.1152/ajpregu.00735.2001

103. Franken P, Gip P, Hagiwara G, Ruby NF, Heller HC. Changes in brain glycogen

after sleep deprivation vary with genotype. Am J Physiol - Regul Integr Comp Physiol. 2003;285: R413–R419. doi:10.1152/ajpregu.00668.2002

104. Franken P, Gip P, Hagiwara G, Ruby NF, Heller HC. Glycogen content in the cerebral cortex increases with sleep loss in C57BL/6J mice. Neurosci Lett. 2006;402: 176–179. doi:10.1016/j.neulet.2006.03.072
105. Nikonova E V, Naidoo N, Zhang L, Romer M, Cater JR, Scharf MT, et al. Changes in components of energy regulation in mouse cortex with increases in wakefulness. Sleep. 2010;33: 889–900. doi:10.1093/sleep/33.7.889
106. Xie L, Kang H, Xu Q, Chen MJ, Liao Y, Thiyagarajan M, et al. Sleep Drives Metabolite Clearance from the Adult Brain. Science (80-). 2013;342: 373–377. doi:10.1126/science.1241224
107. Reimund E. The free radical flux theory of sleep. Med Hypotheses. 1994;43: 231–233. doi:10.1016/0306-9877(94)90071-X
108. Eiland MM, Ramanathan L, Gulyani S, Siegel JM, Eiland MM, Ramanathan L, et al. Increases in amino-cupric-silver staining of the supraoptic nucleus after sleep deprivation. Brain Res. 2002;945: 1–8. doi:10.1016/S0006-8993(02)02448-4
109. Silva RH, Abílio VC, Takatsu AL, Kameda SR, Grassl C, Chehin AB, et al. Role of hippocampal oxidative stress in memory deficits induced by sleep deprivation in mice. Neuropharmacology. 2004;46: 895–903. doi:10.1016/j.neuropharm.2003.11.032
110. D'Almeida V, Lobo LL, Hipólide DC, de Oliveira a C, Nobrega JN, Tufik S. Sleep deprivation induces brain region-specific decreases in glutathione levels. Neuroreport. 1998;9: 2853–2856. doi:10.1097/00001756-199808240-00031

111. Ramanathan L, Gulyani S, Nienhuis R, Siegel JM. Sleep deprivation decreases superoxide dismutase activity in rat hippocampus and brainstem. Neuroreport. 2002;13: 1387–1390. doi:10.1097/00001756-200208070-00007

112. Everson CA, Laatsch CD, Hogg N. Antioxidant defense responses to sleep loss and sleep recovery. Am J Physiol Regul Integr Comp Physiol. 2005;288: R374-383. doi:10.1152/ajpregu.00565.2004

113. D'Almeida V, Hipolide DC, Azzalis LA, Lobo LL, Junqueira VB, Tufik S. Absence of oxidative stress following paradoxical sleep deprivation in rats. Neurosci Lett. 1997;235: 25–28. Available: http://www.ncbi.nlm.nih.gov/pubmed/9389587

114. Cirelli C, Shaw PJ, Rechtschaffen A, Tononi G. No evidence of brain cell degeneration after long-term sleep deprivation in rats. Brain Res. 1999;840: 184–193. doi:10.1016/S0006-8993(99)01768-0

115. Gopalakrishnan A, Ji LL, Cirelli C. Sleep deprivation and cellular responses to oxidative stress. Sleep. 2004;27: 27–35. doi:10.1093/sleep/27.1.27

116. Honda K, Komoda Y, Inoué S. Oxidized glutathione regulates physiological sleep in unrestrained rats. Brain Res. 1994;636: 253–258. doi:10.1016/0006-8993(94)91024-3

117. Ikeda M, Ikeda-Sagara M, Okada T, Clement P, Urade Y, Nagai T, et al. Brain oxidation is an initial process in sleep induction. Neuroscience. 2005;130: 1029–1040. doi:10.1016/j.neuroscience.2004.09.057

118. Koh K, Evans JM, Hendricks JC, Sehgal A. A Drosophila model for age-associated changes in sleep:wake cycles. Proc Natl Acad Sci. 2006;103: 13843–13847. doi:10.1073/pnas.0605903103

119. Williams MJ, Perland E, Eriksson MM, Carlsson J, Erlandsson D, Laan L, et al. Recurrent sleep fragmentation induces insulin and neuroprotective mechanisms in middle-aged flies. Front Aging Neurosci. 2016;8: 1–11. doi:10.3389/fnagi.2016.00180

120. Joiner WJ. Unraveling the Evolutionary Determinants of Sleep. Curr Biol. Elsevier Ltd; 2016;26: R1073–R1087. doi:10.1016/j.cub.2016.08.068

121. Huber R, Hill SL, Holladay C, Biesiadecki M, Tononi G, Cirelli C. Sleep homeostasis in Drosophila melanogaster. Sleep. 2004;27: 628–639. doi:10.1093/sleep/27.4.628

122. Sauer S, Herrmann E, Kaiser W. Sleep deprivation in honey bees. J Sleep Res. 2004;13: 145–152. doi:10.1111/j.1365-2869.2004.00393.x

123. Stephenson R, Chu KM, Lee J. Prolonged deprivation of sleep-like rest raises metabolic rate in the Pacific beetle cockroach, Diploptera punctata (Eschscholtz). J Exp Biol. 2007;210: 2540–2547. doi:10.1242/jeb.005322

124. Ganguly-Fitzgerald I, Donlea J, Shaw PJ. Waking experience affects sleep need in Drosophila. Science (80-). 2006;313: 1775–1781. doi:10.1126/science.1130408

125. Tartar JL, Ward CP, McKenna JT, Thakkar M, Arrigoni E, McCarley RW, et al. Hippocampal synaptic plasticity and spatial learning are impaired in a rat model of sleep fragmentation. Eur J Neurosci. 2006;23: 2739–48. doi:10.1111/j.1460-9568.2006.04808.x

126. Rechtschaffen A, Bergmann BM. Sleep deprivation in the rat: an update of the 1989 paper. Sleep. 2002;25: 18–24.

127. Faraut B, Boudjeltia KZ, Vanhamme L, Kerkhofs M. Immune, inflammatory and cardiovascular consequences of sleep restriction and recovery. Sleep Med Rev. Elsevier Ltd; 2012;16: 137–149. doi:10.1016/j.smrv.2011.05.001

128. Ferrie JE, Kivimaki M, Akbaraly TN, Singh-Manoux A, Miller MA, Gimeno D, et al. Associations Between Change in Sleep Duration and Inflammation: Findings on C-reactive Protein and Interleukin 6 in the Whitehall II Study. Am J Epidemiol. 2013;178: 956–961. doi:10.1093/aje/kwt072

129. Baglioni C, Battagliese G, Feige B, Spiegelhalder K, Nissen C, Voderholzer U, et al. Insomnia as a predictor of depression: A meta-analytic evaluation of longitudinal epidemiological studies. Journal of Affective Disorders. 2011. pp. 10–19. doi:10.1016/j.jad.2011.01.011

130. Hublin C, Partinen M, Koskenvuo M, Kaprio J. Sleep and mortality: A population-based 22-year follow-up study. Sleep. 2007;30: 1245–1253. doi:10.1093/sleep/30.10.1245

131. Gallicchio L, Kalesan B. Sleep duration and mortality: A systematic review and meta-analysis. J Sleep Res. 2009;18: 148–158. doi:10.1111/j.1365-2869.2008.00732.x

132. Gais S. Sleep after learning aids memory recall. Learn Mem. 2006;13: 259–262. doi:10.1101/lm.132106

133. Allada R, Cirelli C, Sehgal A. Molecular mechanisms of sleep homeostasis in flies and mammals. Cold Spring Harb Perspect Biol. 2017;9: 1–20. doi:10.1101/cshperspect.a027730

134. Joiner WJ, Crocker A, White BH, Sehgal A. Sleep in Drosophila is regulated by

adult mushroom bodies. Nature. 2006;441: 757–760. doi:10.1038/nature04811

135. Seidner G, Robinson JE, Wu M, Worden K, Masek P, Roberts SW, et al. Identification of Neurons with a Privileged Role in Sleep Homeostasis in Drosophila melanogaster. Curr Biol. Elsevier Ltd; 2015;25: 2928–2938. doi:10.1016/j.cub.2015.10.006

136. Liu S, Liu Q, Tabuchi M, Wu MN. Sleep drive is encoded by neural plastic changes in a dedicated circuit. Cell. Elsevier Inc.; 2016;165: 1347–1360. doi:10.1016/j.cell.2016.04.013

137. Donlea JM, Alam MN, Szymusiak R. Neuronal substrates of sleep homeostasis; lessons from flies, rats and mice. Curr Opin Neurobiol. Elsevier Ltd; 2017;44: 228–235. doi:10.1016/j.conb.2017.05.003

138. Gopalakrishnan A, Ji LL, Cirelli C. Sleep deprivation and cellular responses to oxidative stress. Sleep J Sleep Sleep Disord Res. 2004;27: 27–35. Available: http://ezproxy.lib.uh.edu/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=psyh&AN=2004-17199-006&site=ehost-live%5Cnhttp://ccirelli@wisc.edu

139. Liu Y, Wheaton AG, Chapman DP, Cunningham TJ, Lu H, Croft JB. Prevalence of Healthy Sleep Duration among Adults — United States, 2014. MMWR Morb Mortal Wkly Rep. 2016;65: 137–141. doi:10.15585/mmwr.mm6506a1

140. Harbison ST, Negron YLS, Hansen NF, Lobell AS. Selection for long and short sleep duration in Drosophila melanogaster reveals the complex genetic network underlying natural variation in sleep. PLoS Genet. 2017;13: e1007098. doi:10.1371/journal.pgen.1007098. eCollection 2017 Dec.

141. Taheri S, Lin L, Austin D, Young T, Mignot E. Short sleep duration is associated

with reduced leptin, elevated ghrelin, and increased body mass index. PLoS Med. 2004;1: 210–217. doi:10.1371/journal.pmed.0010062

142. Wang Y, Carreras A, Lee S, Hakim F, Zhang SX, Nair D, et al. Chronic sleep fragmentation promotes obesity in young adult mice. Obesity. 2014;22: 758–762. doi:10.1002/oby.20616

143. Masek P, Reynolds LA, Bollinger WL, Moody C, Mehta A, Murakami K, et al. Altered regulation of sleep and feeding contributes to starvation resistance in Drosophila melanogaster. J Exp Biol. 2014;217: 3122–3132. doi:10.1242/jeb.103309

144. Prather AA, Janicki-Deverts D, Hall MH, Cohen S. Behaviorally Assessed Sleep and Susceptibility to the Common Cold. Sleep. 2015;38: 1353–1359. doi:10.5665/sleep.4968

145. Irwin MR, McClintick J, Costlow C, Fortner M, White J, Gillin JC. Partial night sleep deprivation reduces natural killer and cellular immune responses in humans. FASEB J. 1996;10: 643–653.

146. Lungato L, Gazarini ML, Paredes-Gamero EJ, Tufik S, D'Almeida V. Paradoxical sleep deprivation impairs mouse survival after infection with malaria parasites. Malar J. 2015;14. doi:10.1186/s12936-015-0690-7

147. Zager a, Andersen ML, Ruiz FS, Antunes IB, Tufik S. Effects of acute and chronic sleep loss on immune modulation of rats. Am J Physiol Regul Integr Comp Physiol. 2007;293: R504-9. doi:10.1152/ajpregu.00105.2007

148. Cochemé HM, Murphy MP. Complex I is the major site of mitochondrial superoxide production by paraquat. J Biol Chem. 2008;283: 1786–1798.

doi:10.1074/jbc.M708597200

149. Naidoo N, Giang W, Galante RJ, Pack AI. Sleep deprivation induces the unfolded protein response in mouse cerebral cortex. J Neurochem. 2005;92: 1150–1157. doi:10.1111/j.1471-4159.2004.02952.x

150. Naidoo N, Casiano V, Cater J, Zimmerman J, Pack AI. A role for the molecular chaperone protein BiP/GRP78 in Drosophila sleep horneostasis. Sleep. 2007;30: 557–565.

151. Brown MK, Strus E, Naidoo N. Reduced sleep during social isolation leads to cellular stress and induction of the unfolded protein response. Sleep. 2017;40. doi:10.1093/sleep/zsx095

152. Tang S, Le PK, Tse S, Wallace DC, Huang T. Heterozygous mutation of Opa1 in Drosophila shortens lifespan mediated through increased reactive oxygen species production. PLoS One. 2009;4. doi:10.1371/journal.pone.0004492

153. Yang Y, Gehrke S, Haque ME, Imai Y, Kosek J, Yang L, et al. Inactivation of Drosophila DJ-1 leads to impairments of oxidative stress response and phosphatidylinositol 3-kinase/Akt signaling. Proc Natl Acad Sci U S A. 2005;102: 13670–13675. doi:10.1073/pnas.0504610102

154. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Curr Biol. Elsevier; 2014;24: R453–R462. doi:10.1016/j.cub.2014.03.034

155. Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, et al. ROS signaling: The new wave? Trends Plant Sci. Elsevier Ltd; 2011;16: 300–309. doi:10.1016/j.tplants.2011.03.007

156. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circulation

Research. 2010. pp. 1058–1070. doi:10.1161/CIRCRESAHA.110.223545

157. Kayama Y, Raaz U, Jagger A, Adam M, Schellinger IN, Sakamoto M, et al. Diabetic cardiovascular disease induced by oxidative stress. International Journal of Molecular Sciences. 2015. pp. 25234–25263. doi:10.3390/ijms161025234

158. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. Am J Cardiol. 2003;91: 7–11. doi:10.1016/S0002-9149(02)03144-2

159. Youn J-YY, Siu KL, Lob HE, Itani H, Harrison DG, Cai H. Role of vascular oxidative stress in obesity and metabolic syndrome. Diabetes. 2014;63: 2344–2355. doi:10.2337/db13-0719

160. Carvajal K, Balderas-Villalobos J, Bello-Sanchez MD, Phillips-Farfán B, Molina-Muñoz T, Aldana-Quintero H, et al. Ca2+ mishandling and cardiac dysfunction in obesity and insulin resistance: Role of oxidative stress. Cell Calcium. 2014. pp. 408–415. doi:10.1016/j.ceca.2014.08.003

161. Sterniczuk R, Theou O, Rusak B, Rockwood K. Sleep Disturbance is Associated with Incident Dementia and Mortality. Curr Alzheimer Res. 2013;10: 767–775. doi:10.2174/15672050113109990134

162. Schenck CH, Boeve BF, Mahowald MW. Delayed emergence of a parkinsonian disorder or dementia in 81% of older men initially diagnosed with idiopathic rapid eye movement sleep behavior disorder: A 16-year update on a previously reported series. Sleep Med. 2013;14: 744–748. doi:10.1016/j.sleep.2012.10.009

163. Lazar AS, Panin F, Goodman AOG, Lazic SE, Lazar ZI, Mason SL, et al. Sleep deficits but no metabolic deficits in premanifest Huntington's disease. Ann Neurol.

2015;78: 630–648. doi:10.1002/ana.24495

164. Allan Butterfield D, Castegna A, Lauderback CM, Drake J. Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. Neurobiology of Aging. 2002. pp. 655–664. doi:10.1016/S0197-4580(01)00340-2

165. Dexter DT, Carter CJ, Wells FR, Javoy-Agid F, Agid Y, Lees A, et al. Basal Lipid Peroxidation in Substantia Nigra Is Increased in Parkinson's Disease. J Neurochem. 1989;52: 381–389. doi:10.1111/j.1471-4159.1989.tb09133.x

166. Sorolla MA, Reverter-Branchat G, Tamarit J, Ferrer I, Ros J, Cabiscol E. Proteomic and oxidative stress analysis in human brain samples of Huntington disease. Free Radic Biol Med. 2008;45: 667–678. doi:10.1016/j.freeradbiomed.2008.05.014

167. Cirelli C, Tononi G. Gene expression in the brain across the sleep-waking cycle. Brain Res. 2000;885: 303–321. doi:10.1016/S0006-8993(00)03008-0

168. Terao A, Steininger TL, Hyder K, Apte-Deshpande A, Ding J, Rishipathak D, et al. Differential increase in the expression of heat shock protein family members during sleep deprivation and during sleep. Neuroscience. 2003;116: 187–200. doi:10.1016/S0306-4522(02)00695-4

169. Owusu-Ansah E, Yavari A, Banerjee U. A protocol for in vivo detection of reactive oxygen species. Protoc Exch. 2008; doi:10.1038/nprot.2008.23

170. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003;552: 335–344. doi:10.1111/j.1469-7793.2003.00335.x

171. Yagi K. Simple assay for the level of total lipid peroxides in serum or plasma.

Methods Mol Biol. 1998;108: 101–106. doi:10.1385/0-89603-472-0:101

172. Luo Y, Hawkley LC, Waite LJ, Cacioppo JT. Loneliness, health, and mortality in old age: A national longitudinal study. Soc Sci Med. 2012;74: 907–914. doi:10.1016/j.socscimed.2011.11.028

173. McPherson M, Smith-Lovin L, Brashears ME. Social Isolation in America: Changes in Core Discussion Networks over Two Decades. Am Sociol Rev. 2008;73: 1022–1022. doi:10.1177/000312240807300610

174. Patterson A, Veenstra G. Loneliness and risk of mortality: a longitudinal investigation in Alameda County, California. Soc Sci Med. 2010;71: 181–186. doi:10.1016/j.socscimed.2010.03.024

175. Hawkley LC, Cacioppo JT. Loneliness and pathways to disease. Brain, Behavior, and Immunity. 2003. doi:10.1016/S0889-1591(02)00073-9

176. House JS, Landis KR, Umberson D. Social relationships and health. [Internet]. Science. 1988. pp. 540–545. doi:10.1126/science.3399889

177. Cacioppo JT, Hawkley LC, Crawford LE, Ernst JM, Burleson MH, Kowalewski RB, et al. Loneliness and Health: Potential Mechanisms. Psychosom Med. 2002;64: 407–417. doi:10.1097/00006842-200205000-00005

178. Kiecolt-Glaser JK, Garner W, Speicher C, Penn GM, Holliday J, Glaser R. Psychosocial modifiers of immunocompetence in medical students. Psychosom Med. 1984;46: 7–14. doi:10.1097/00006842-198401000-00003

179. Glaser R, Kiecolt-Glaser JK, Speicher CE, Holliday JE. Stress, loneliness, and changes in herpesvirus latency. J Behav Med. 1985;8: 249–260. doi:10.1007/BF00870312

180. Kiecolt-Glaser JK, Ricker D, George J, Messick G, Speicher CE, Garner W, et al. Urinary cortisol levels, cellular immunocompetency, and loneliness in psychiatric inpatients. Psychosom Med. 1984;46: 15–23. doi:10.1097/00006842-198401000-00004

181. Pressman SD, Cohen S, Miller GE, Barkin A, Rabin BS, Treanor JJ. Loneliness, Social Network Size, and Immune Response to Influenza Vaccination in College Freshmen. Heal Psychol. 2005;24: 297–306. doi:10.1037/0278-6133.24.3.297

182. Nonogaki K, Nozue K, Oka Y. Social isolation affects the development of obesity and type 2 diabetes in mice. Endocrinology. 2007;148: 4658–4666. doi:10.1210/en.2007-0296

183. Poletto R, Steibel JP, Siegford JM ZA. Effects of early weaning and social isolation on the expression of glucocorticoid and mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase 1 and 2 mRNAs in the frontal cortex and hippocampus of piglets. Brain Res. 2006;1067: 36–41.

184. Kanitz E, Tuchscherer M, Puppe B, Tuchscherer A, Stabenow B. Consequences of repeated early isolation in domestic piglets (Sus scrofa) on their behavioural, neuroendocrine, and immunological responses. Brain Behav Immun. 2004;18: 35–45. doi:10.1016/S0889-1591(03)00085-0

185. Nation DA, Gonzales JA, Mendez AJ, Zaias J, Szeto A, Brooks LG, et al. The effect of social environment on markers of vascular oxidative stress and inflammation in the Watanabe heritable hyperlipidemic rabbit. Psychosom Med. 2008;70: 269–275. doi:10.1097/PSY.0b013e3181646753

186. Lyons DM, Ha CMG, Levine S. Social effects and circadian rhythms in squirrel

monkey pituitary-adrenal activity. Horm Behav. 1995;29: 177–190. doi:10.1006/hbeh.1995.1013

187. Yeh SR, Fricke R a, Edwards DH. The effect of social experience on serotonergic modulation of the escape circuit of crayfish. Science. 1996;271: 366–369. doi:10.1126/science.271.5247.366

188. Alexander R. Social Behavior in crickets. Behaviour. 1961;17: 130223.

189. Breed MD. Correlations between aggressiveness and corpora allata volume, social isolation, age and dietary protein in worker honeybees. Insectes Soc. 1983;30: 482–495. doi:10.1007/BF02223979

190. Pfennig DW, Reeve HK. Neighbor Recognition and Context-dependent Aggression in a Solitary Wasp, Sphecius speciosus (Hymenoptera: Sphecidae). Ethology. 1989;80: 1–18. doi:10.1111/j.1439-0310.1989.tb00726.x

191. Hoffmann A a. The influence of age and experience with conspecifics on territorial behavior inDrosophila melanogaster. J Insect Behav. 1990;3: 1–12. doi:10.1007/BF01049191

192. Zhou C, Rao Y, Rao Y. A subset of octopaminergic neurons are important for Drosophila aggression. Nat Neurosci. 2008;11: 1059–1067. doi:10.1038/nn.2164

193. Ueda A, Kidokoro Y. Aggressive behaviours of female Drosophila melanogaster are influenced by their social experience and food resources. Physiol Entomol. 2002;27: 21–28. doi:10.1046/j.1365-3032.2002.00262.x

194. Long DB. EFFECTS OF POPULATION DENSITY ON LARVAE OF LEPIDOPTERA. Trans R Entomol Soc London. 1953;104: 543–585. doi:10.1111/j.1365-2311.1953.tb01247.x

195. Pener MP, Yerushalmi Y. The physiology of locust phase polymorphism: An update. Journal of Insect Physiology. 1998. pp. 365–377. doi:10.1016/S0022-1910(97)00169-8

196. Donlea JM, Shaw PJ. Sleeping together using social interactions to understand the role of sleep in plasticity. Advances in genetics. 2009. pp. 57–81. doi:10.1016/S0065-2660(09)68003-2

197. Technau GM. Fiber number in the mushroom bodies of adult Drosophila melanogaster depends on age, sex and experience. J Neurogenet. 2007;21: 183–196. doi:10.1080/01677060701695359

198. Ueda A, Wu CF. Effects of social isolation on neuromuscular excitability and aggressive behaviors in drosophila: Altered responses by Hk and gsts1, two mutations implicated in redox regulation. J Neurogenet. 2009;23: 378–394. doi:10.3109/01677060903063026

199. Starks PT, Blackie CA, Thomas D Seeley PT. Fever in honeybee colonies. Naturwissenschaften. 2000;87: 229–231. doi:10.1007/s001140050709

200. Little AE., Murakami T, Mueller UG, Currie CR. Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. Biol Lett. 2006;2: 12–16. doi:10.1098/rsbl.2005.0371

201. Trumbo ST, Huang ZY, Robinson GE. Division of labor between undertaker specialists and other middle-aged workers in honey bee colonies. Behav Ecol Sociobiol. 1997;41: 151–163. doi:10.1007/s002650050374

202. Christe P, Oppliger A, Bancalà F, Castella G, Chapuisat M. Evidence for collective medication in ants. Ecol Lett. 2003;6: 19–22. doi:10.1046/j.1461-

0248.2003.00395.x

203. Jiang Z, Rompala GR, Zhang S, Cowell RM, Nakazawa K. Social isolation exacerbates schizophrenia-like phenotypes via oxidative stress in cortical interneurons. Biol Psychiatry. 2013;73: 1024–1034. doi:10.1016/j.biopsych.2012.12.004

204. Naidoo N, Casiano V, Cater J, Zimmerman J, Pack AI. A role for the molecular chaperone protein BiP/GRP78 in Drosophila sleep homeostasis. Sleep. 2007;30: 557–565.

205. Brown MK, Naidoo N. The UPR and the anti-oxidant response: Relevance to sleep and sleep loss. Mol Neurobiol. 2010;42: 103–113. doi:10.1007/s12035-010-8114-8

206. Jovaisaite V, Mouchiroud L, Auwerx J. The mitochondrial unfolded protein response, a conserved stress response pathway with implications in health and disease. J Exp Biol. 2014;217: 137–143. doi:10.1242/jeb.090738

207. Keebaugh ES, Park JH, Su C, Yamada R, Ja WW. Nutrition Influences Caffeine-Mediated Sleep Loss in Drosophila. Sleep. 2017;40. doi:10.1093/sleep/zsx146

208. Yamazaki M, Tomita J, Takahama K, Ueno T, Mitsuyoshi M, Sakamoto E, et al. High calorie diet augments age-associats sleep impairment in Drosophila. Biochem Biophys Res Commun. 2012;417: 812–816. doi:10.1016/j.bbrc.2011.12.041

209. Tang H. Regulation and function of the melanization reaction in Drosophila. Fly. 2009. pp. 105–111. doi:10.4161/fly.3.1.7747

210. Ayres JS, Schneider DS. A Signaling Protease Required for Melanization in

Drosophila Affects Resistance and Tolerance of Infections. PLoS Biol. 2008;6: e305. doi:10.1371/journal.pbio.0060305

Appendix I: *fumin* Lifespan

Though it was originally published that *fumin* mutants have a normal lifespan [38], I found that they have a shortened lifespan (Fig. 1.1). Based on another report which found that caloric content of food can influence sleep amount as well as lifespan in *fumin* mutants [208], I suspect that this difference in lifespan is due to a difference in food.

Yamazaki et al. found that, when fed a high calorie diet, *fumin* mutants sleep even less than originally reported by Kume [38] and have a shortened lifespan. This result is consistent with my observation: I found that *fumin* mutants sleep 95% less than controls (Fig. 2.5C, left panel) rather than the ~66% reduction originally reported, and I found that these extremely short-sleeping mutants have a significantly shortened lifespan (Fig. 1.1).

We recently discovered that the molasses food used to raise these flies at the time of this experiment contained Blackstrap Molasses, which has an unusually high sugar content. Taken together, I suspect that the more dramatic sleep phenotype and shortened lifespan that I observed in *fumin* mutants may have been due to a high calorie diet.

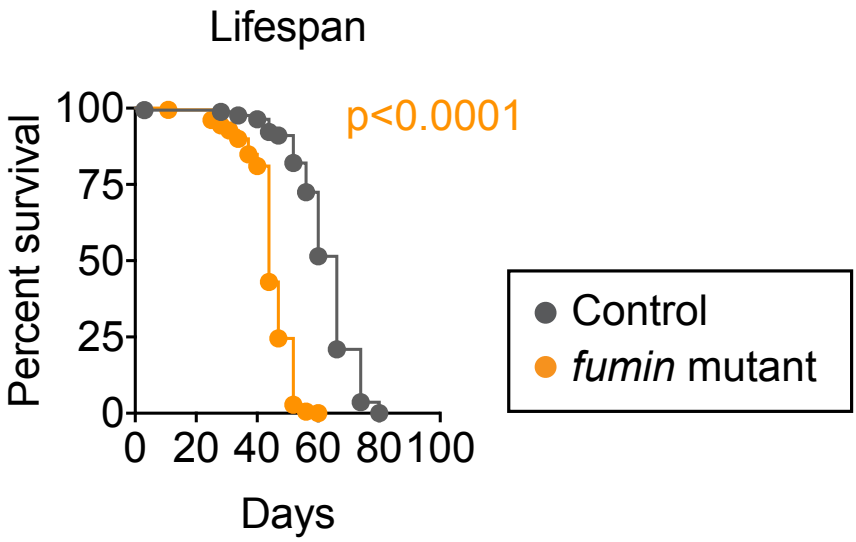

Fig. 1.1. *fumin* mutants have a shortened lifespan.
Short-sleeping *fumin* mutants have a shortened lifespan compared to controls ($p<0.0001$, n=167-179 flies/genotype). p-value was obtained by log-rank analysis. Experiment was performed once.

Appendix II: Melanization Data

Innate immunity in the fly is characterized by three main immune responses, one of which is melanization, a process that produces brown deposits of melanin to help sequester bacteria at the site of a wound. Melanization requires the activation of an enzymatic cascade that produces ROS and other cytotoxic intermediates which aid in killing pathogens [209]. Particular infections, such as *Salmonella typhimurium* and *Listeria monocytogenes,* have been shown to induce a systemic melanization response in the fly [210]. This systemic response occurs days after the initial infection and is characterized by the formation of melanin deposits under the cuticle in various areas of the body.

I observed increased systemic melanization in short-sleeping neuronal *inc-RNAi* flies relative to controls after injection with *Salmonella typhimurium* (Fig. 3.1A). This systemic response was separate from wounding induced melanization, which I found to be comparable in neuronal *inc-RNAi* flies and controls at the injection site. Interestingly, I observed the opposite phenotype in long-sleeping *dFMR1* mutants after injection with *Listeria monocytogenes* (Fig 3.1B). In this case, *dFRM1* flies had reduced systemic melanization relative to controls, but comparable melanization at the wound site.

Though ROS is thought to be a byproduct of melanization rather than an activator of it, the melanization cascade is triggered by oxidation of the enzyme phenoloxidase; thus, differences in redox state in the body could cause early activation or a delay in the systemic melanization response. If ROS levels are high in neuronal *inc-RNAi* flies, this could contribute to their increased systemic melanization response.

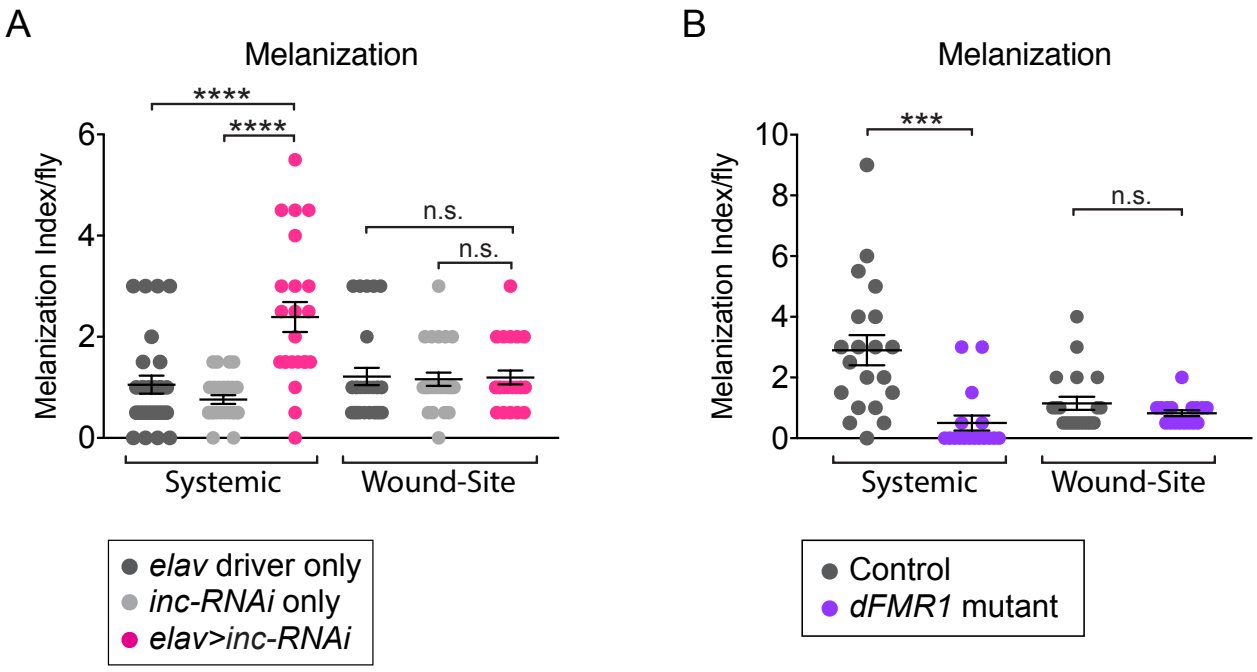

Fig. 3.1. Melanization in short-sleeping and long-sleeping flies.
Short-sleeping neuronal *inc-RNAi* flies exhibit a more robust systemic melanization response than controls after injection with *Salmonella typhimurium* (A, $p<0.0001$ relative to either control, n=23-28 flies/genotype). As a control, melanization at the injection wound-site was confirmed to be comparable between neuronal *inc-RNAi* flies and controls ($p>0.05$ relative to either control), indicating that the altered system melanization is in reponse to the infection rather than wounding. Long-sleeping *dFMR1* flies exhibit less robust systemic melanization than controls after injection with *Listeria monocytogenes* (B, $p<0.001$, n=17-21 flies/genotype), but have comparable wounding site melanization relative to controls ($p>0.05$). Data are shown as mean ± SEM. Each data point represents the melanization index of an individual fly. Melanization index was calculated based on the size and number of melanin spots on each fly. p-values were obtained by ordinary one-way ANOVA followed by a Tukey post hoc test to correct for multiple comparisons (A) or by students unpaired t test (B). Data from representative experiments are shown. Experiments were performed at least twice.

Appendix III: Stress Response Mutants

Mutant	Repeats	Sleep	Bout Length	Bout Number
cat	3	↓↓	↓↓↓	↑↑↑
cat/iso	1			↑
GSTD1	4	↓↓		
GSTS1	4	↓↓↓↓	↓↓↓	↑↑↑
GSTS1^{k}	4	↓↓↓↓	↓↓↓↓	↑↑↑
hsp60	4	↓↓↓	↓↓↓	↑↑↑
hsp70	4	↓↓↓	↓	
keap1	6	↓↓↓↓	↓↓	
nrf2	5	↓↓↓↓↓	↓↓↓↓	↑↑↑
PERK	5	↓↓↓	↓↓	↑

Table 3.1. The sleep profiles of a number of mutants, all with defects in genes that are induced by ROS, are shown. The mutants exhibited a strikingly similar pattern. The number of total experimental trials is listed to the left. Arrows indicate either a significant increase or a significant decrease compared to controls in an independent trial. Trials resulting in no significant differences are not shown.

	H2O2 Feeding		PQ Injection	
Mutant	1%	4%	3 mM	4 mM
Cat/iso		S		
GSTD1		R	Nd	R
GSTS1	R	S S	Nd	Nd
GSTS1k	R	Nd Nd	S	Nd
hsp60	R	S R		
hsp70	S	S S	Nd	R R S Nd
keap1	S			
nrf2	S	S S	R R	R R R S
PERK		S		

Table 3.2. Stress response mutants have varied responses to oxidative stress. Stress response mutants were subjected to H_2O_2 feeding (1% or 4%) or paraquat (PQ) injection (3mM or 4mM). Individual trials are represented as either R (resistant compared to control), S (sensitive compared to control) or Nd (no difference).